



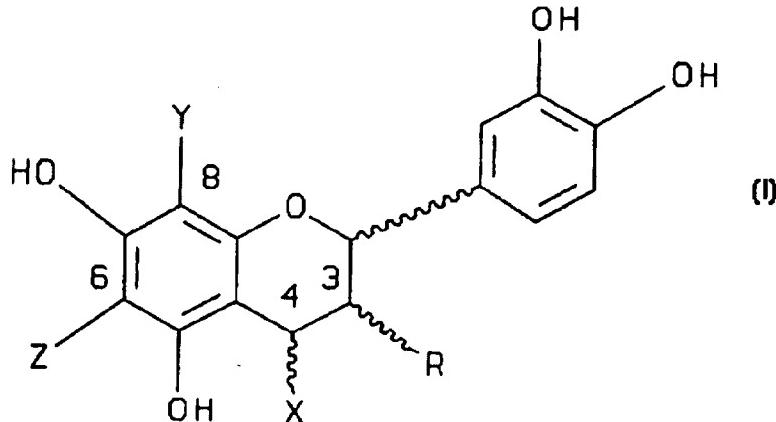
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(54) Title: COCOA EXTRACT COMPOUNDS AND METHODS FOR MAKING AND USING THE SAME

(57) Abstract

Disclosed and claimed are cocoa extracts, compounds, combinations thereof and compositions containing the same, such as polyphenols or procyandins, methods for preparing such extracts, compounds and compositions, as well as uses for them, especially a polymeric compound of the formula A_n, wherein A is a monomer of formula (I) wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units; R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar or 3-(β)-O-sugar; bonding between adjacent monomers takes place at positions 4, 6 or 8; a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry; X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto (the bonding of the additional monomeric unit adjacent to the terminal monomeric unit) is at position 4 and optionally Y = Z = hydrogen; the sugar is optionally substituted with a phenolic moiety, at any position on the sugar, for instance via an ester bond; and pharmaceutically acceptable salts or derivatives thereof (including oxidation products).



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**COCOA EXTRACT COMPOUNDS AND METHODS
FOR MAKING AND USING THE SAME**

REFERENCE TO RELATED APPLICATION

5 Reference is made to copending U.S. application Nos. 08/709,406, filed September 6, 1996, 08/631,661, filed April 2, 1996, and 08/317,226, filed October 3, 1994 (now U.S. Patent No. 5,554,645) and PCT/US96/04497, each of which is incorporated herein by reference.

10 FIELD OF THE INVENTION

This invention relates to cocoa extracts and compounds therefrom such as polyphenols preferably polyphenols enriched with procyanidins. This invention also relates to methods for preparing such extracts and 15 compounds, as well as to uses for them; for instance, as antineoplastic agents, antioxidants, DNA topoisomerase II enzyme inhibitors, cyclo-oxygenase and/or lipoxygenase modulators, NO (Nitric Oxide) or NO-synthase modulators, as non-steroidal antiinflammatory agents, apoptosis 20 modulators, platelet aggregation modulators, blood or *in vivo* glucose modulators, antimicrobials, and inhibitors of oxidative DNA damage.

Documents are cited in this disclosure with a full citation for each appearing thereat or in a 25 References section at the end of the specification, preceding the claims. These documents pertain to the field of this invention; and, each document cited herein is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

30 Polyphenols are an incredibly diverse group of compounds (Ferreira et al., 1992) which widely occur in a variety of plants, some of which enter into the food chain. In some cases they represent an important class of compounds for the human diet. Although some of the 35 polyphenols are considered to be nonnutritive, interest in these compounds has arisen because of their possible beneficial effects on health.

For instance, quercetin (a flavonoid) has been shown to possess anticarcinogenic activity in

experimental animal studies (Deshner et al., 1991 and Kato et al., 1983). (+)-Catechin and (-)-epicatechin (flavan-3-ols) have been shown to inhibit Leukemia virus reverse transcriptase activity (Chu et al., 1992).

5 Nobotanin (an oligomeric hydrolyzable tannin) has also been shown to possess anti-tumor activity (Okuda et al., 1992). Statistical reports have also shown that stomach cancer mortality is significantly lower in the tea producing districts of Japan. Epigallocatechin gallate

10 has been reported to be the pharmacologically active material in green tea that inhibits mouse skin tumors (Okuda et al., 1992). Ellagic acid has also been shown to possess anticarcinogen activity in various animal tumor models (Bukharta et al., 1992). Lastly,

15 proanthocyanidin oligomers have been patented by the Kikkoman Corporation for use as antimutagens. Indeed, the area of phenolic compounds in foods and their modulation of tumor development in experimental animal models has been recently presented at the 202nd National

20 Meeting of The American Chemical Society (Ho et al., 1992; Huang et al., 1992).

However, none of these reports teaches or suggests cocoa extracts or compounds therefrom, any methods for preparing such extracts or compounds

25 therefrom, or, any uses for cocoa extracts or compounds therefrom, as antineoplastic agents, antioxidants, DNA topoisomerase II enzyme inhibitors, cyclo-oxygenase and/or lipoxygenase modulators, NO (Nitric Oxide) or NO-synthase modulators, as non-steroidal antiinflammatory

30 agents, apoptosis modulators, platelet aggregation modulators, blood or *in vivo* glucose modulators, antimicrobials, or inhibitors of oxidative DNA damage.

OBJECTS AND SUMMARY OF THE INVENTION

Since unfermented cocoa beans contain

35 substantial levels of polyphenols, the present inventors considered it possible that similar activities of and uses for cocoa extracts, e.g., compounds within cocoa,

could be revealed by extracting such compounds from cocoa and screening the extracts for activity. The National Cancer Institute has screened various *Theobroma* and *Herrania* species for anti-cancer activity as part of their massive natural product selection program. Low levels of activity were reported in some extracts of cocoa tissues, and the work was not pursued. Thus, in the antineoplastic or anti-cancer art, cocoa and its extracts were not deemed to be useful; i.e., the teachings in the antineoplastic or anti-cancer art lead the skilled artisan away from employing cocoa and its extracts as cancer therapy.

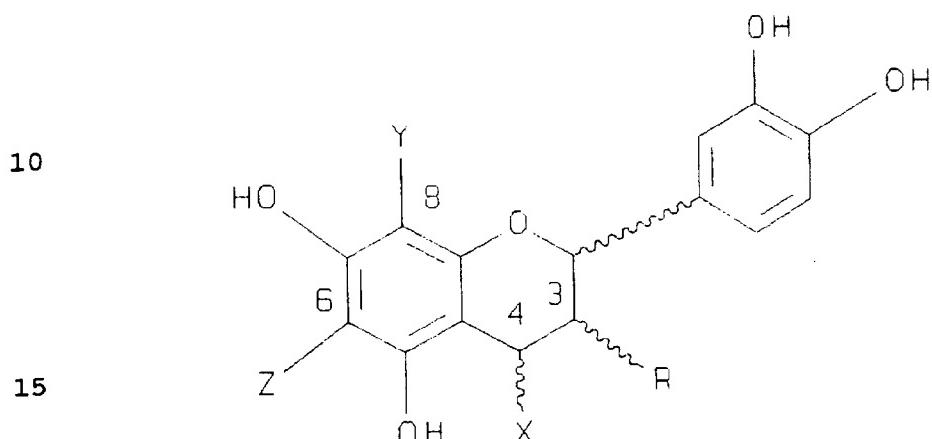
Since a number of analytical procedures were developed to study the contributions of cocoa polyphenols to flavor development (Clapperton et al., 1992), the present inventors decided to apply analogous methods to prepare samples for anti-cancer screening, contrary to the knowledge in the antineoplastic or anti-cancer art. Surprisingly, and contrary to the knowledge in the art, e.g., the National Cancer Institute screening, the present inventors discovered that cocoa polyphenol extracts which contain procyanidins, have significant utility as anti-cancer or antineoplastic agents.

Additionally, the inventors demonstrate that cocoa extracts containing procyanidins and compounds from cocoa extracts have utility as antineoplastic agents, antioxidants, DNA topoisomerase II enzyme inhibitors, cyclo-oxygenase and/or lipoxygenase modulators, NO (Nitric Oxide) or NO-synthase modulators, as non-steroidal antiinflammatory agents, apoptosis modulators, platelet aggregation modulators, blood or *in vivo* glucose modulators, antimicrobials, and inhibitors of oxidative DNA damage.

It is an object of the present invention to provide a method for producing cocoa extract and/or compounds therefrom.

It is another object of the invention to provide a cocoa extract and/or compounds therefrom.

It is still another object of the present invention to provide a polymeric compound of the formula 5 A_n, wherein A is a monomer having the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions 4, 6 or 8;

a bond of an additional monomeric unit in position 4 has α or β stereochemistry;

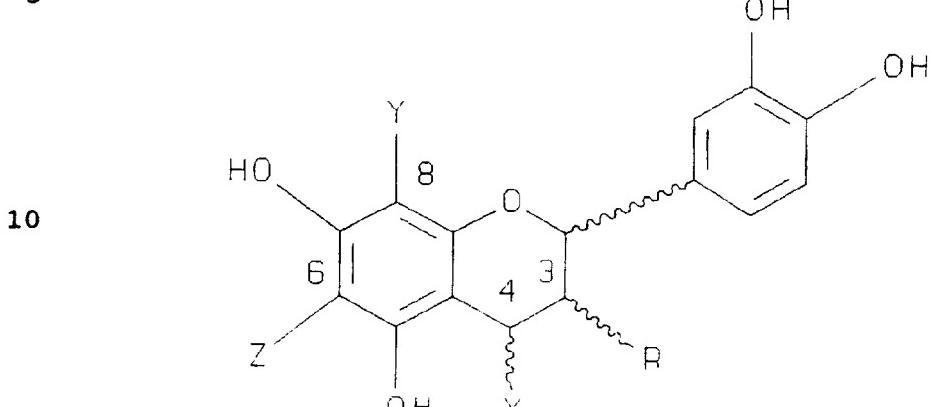
X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety at any position, for instance, via an ester bond,

and pharmaceutically acceptable salts or derivatives thereof (including oxidation products).

It is still a further object of the present invention to provide a polymeric compound of the formula A_n, wherein A is a monomer having the formula:

5



10

15

wherein n is an integer from 2 to 18, e.g., 3 to 18;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

20

adjacent monomers bind at position 4 by (4 \rightarrow 6) or (4 \rightarrow 8);

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen;

a bond at position 4 has α or β stereochemistry;

the sugar is optionally substituted with a phenolic moiety at any position, for instance, via an ester bond,

and pharmaceutically acceptable salts or derivatives thereof (including oxidation products).

It is another object of the invention to provide an antioxidant composition.

It is another object of the invention to demonstrate inhibition of DNA topoisomerase II enzyme activity.

It is yet another object of the present 5 invention to provide a method for treating tumors or cancer.

It is still another object of the invention to provide an anti-cancer, anti-tumor or antineoplastic compositions.

10 It is still a further object of the invention to provide an antimicrobial composition.

It is yet another object of the invention to provide a cyclo-oxygenase and/or lipoxygenase modulating composition.

15 It is still another object of the invention to provide an NO or NO-synthase-modulating composition.

It is a further object of the invention to provide a non-steroidal antiinflammatory composition.

It is another object of the invention to 20 provide a blood or *in vivo* glucose-modulating composition.

It is yet a further object of the invention to provide a method for treating a patient with an antineoplastic, antioxidant, antimicrobial, cyclo- 25 oxygenase and/or lipoxygenase modulating or NO or NO-synthase modulating non-steroidal antiinflammatory modulating and/or blood or *in vivo* glucose-modulating composition.

It is an additional object of the invention to 30 provide compositions and methods for inhibiting oxidative DNA damage.

It is yet an additional object of the invention to provide compositions and methods for platelet aggregation modulation.

35 It is still a further object of the invention to provide compositions and methods for apoptosis modulation.

It is a further object of the invention to provide a method for making any of the aforementioned compositions.

And, it is an object of the invention to 5 provide a kit for use in the aforementioned methods or for preparing the aforementioned compositions.

It has been surprisingly discovered that cocoa extract, and compounds therefrom, have anti-tumor, anti-cancer or antineoplastic activity or, is an antioxidant 10 composition or, inhibits DNA topoisomerase II enzyme activity or, is an antimicrobial or, is a cyclo-oxygenase and/or lipoxygenase modulator or, is a NO or NO-synthase modulator, is a non-steroidal antiinflammatory agent, apoptosis modulator, platelet aggregation modulator or, 15 is a blood or *in vivo* glucose modulator, or is an inhibitor of oxidative DNA damage.

Accordingly, the present invention provides a substantially pure cocoa extract and compounds therefrom. The extract or compounds preferably comprises 20 polyphenol(s) such as polyphenol(s) enriched with cocoa procyanidin(s), such as polyphenols of at least one cocoa procyanidin selected from (-) epicatechin, (+) catechin, procyanidin B-2, procyanidin oligomers 2 through 18, e.g., 3 through 18, such as 2 through 12 or 3 through 12, 25 preferably 2 through 5 or 4 through 12, more preferably 3 through 12, and most preferably 5 through 12, procyanidin B-5, procyanidin A-2 and procyanidin C-1.

The present invention also provides an anti-tumor, anti-cancer or antineoplastic or antioxidant or 30 DNA topoisomerase II inhibitor, or antimicrobial, or cyclo-oxygenase and/or lipoxygenase modulator, or an NO or NO-synthase modulator, nonsteroidal antiinflammatory agent, apoptosis modulator, platelet aggregation modulator, blood or *in vivo* glucose modulator, or 35 oxidative DNA damage inhibitory composition comprising a substantially pure cocoa extract or compound therefrom or synthetic cocoa polyphenol(s) such as polyphenol(s)

enriched with procyanidin(s) and a suitable carrier, e.g., a pharmaceutically, veterinary or food science acceptable carrier. The extract or compound therefrom preferably comprises cocoa procyanidin(s). The cocoa extract or compounds therefrom is preferably obtained by a process comprising reducing cocoa beans to powder, defatting the powder and, extracting and purifying active compound(s) from the powder.

The present invention further comprehends a method for treating a patient in need of treatment with an anti-tumor, anti-cancer, or antineoplastic agent or an antioxidant, or a DNA topoisomerase II inhibitor, or antimicrobial, or cyclo-oxygenase and/or lipoxygenase modulator, or an NO or NO-synthase modulator, non-steroidal antiinflammatory agent, apoptosis modulator, platelet aggregation modulator, blood or *in vivo* glucose modulator or inhibitor of oxidative DNA damage, comprising administering to the patient a composition comprising an effective quantity of a substantially pure cocoa extract or compound therefrom or synthetic cocoa polyphenol(s) or procyanidin(s) and a carrier, e.g., a pharmaceutically, veterinary or food science acceptable carrier. The cocoa extract or compound therefrom can be cocoa procyanidin(s); and, is preferably obtained by reducing cocoa beans to powder, defatting the powder and, extracting and purifying active compound(s) from the powder.

Additionally, the present invention provides a kit for treating a patient in need of treatment with an anti-tumor, anti-cancer, or antineoplastic agent or antioxidant or DNA topoisomerase II inhibitor, or antimicrobial, or cyclo-oxygenase and/or lipoxygenase modulator, or an NO or NO-synthase modulator, non-steroidal antiinflammatory agent, apoptosis modulator, platelet aggregation modulator inhibitor of oxidative DNA damage, or blood or *in vivo* glucose modulator comprising a substantially pure cocoa extract or compounds therefrom.

or synthetic cocoa polyphenol(s) or procyanidin(s) and a suitable carrier, e.g., a pharmaceutically, veterinary or food science acceptable carrier, for admixture with the extract or compound therefrom or synthetic polyphenol(s) 5 or procyanidin(s).

The present invention provides compounds as illustrated in Figs. 38A to 38P and 39A to 39AA; and linkages of 4→6 and 4→8 are presently preferred.

The invention even further encompasses food 10 preservation or preparation compositions comprising an inventive compound, and methods for preparing or preserving food by adding the composition to food.

And, the invention still further encompasses a DNA topoisomerase II inhibitor comprising an inventive 15 compound and a suitable carrier or diluent, and methods for treating a patient in need of such treatment by administration of the composition.

Considering broadly the aforementioned 20 embodiments involving cocoa extracts, the invention also includes such embodiments wherein an inventive compound is used instead of or as the cocoa extracts. Thus, the invention comprehends kits, methods, and compositions analogous to those above-stated with regard to cocoa extracts and with an inventive compound.

25 These and other objects and embodiments are disclosed or will be obvious from the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following Detailed Description will be 30 better understood by reference to the accompanying drawings wherein:

Fig. 1 shows a representative gel permeation chromatogram from the fractionation of crude cocoa procyanidins;

35 Fig. 2A shows a representative reverse-phase HPLC chromatogram showing the separation (elution

profile) of cocoa procyanidins extracted from unfermented cocoa;

Fig. 2B shows a representative normal phase HPLC separation of cocoa procyanidins extracted from 5 unfermented cocoa;

Fig. 3 shows several representative procyanidin structures;

Figs. 4A-4E show representative HPLC chromatograms of five fractions employed in screening for 10 anti-cancer or antineoplastic activity;

Figs. 5 and 6A-6D show the dose-response relationship between cocoa extracts and cancer cells ACHN (Fig. 5) and PC-3 (Figs. 6A-6D) (fractional survival vs. dose, $\mu\text{g/mL}$); M&M2 F4/92, M&MA+E U12P1, M&MB+E Y192P1, 15 M&MC+E U12P2, M&MD+E U12P2;

Figs. 7A to 7H show the typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, A+B, A+E, and A+D, and the PC-3 cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1A 0212P3, MM-1 20 B 0162P1, MM-1 C 0122P3, MM-1 D 0122P3, MM-1 E 0292P8, MM-1 A/B 0292P6, MM-1 A/E 0292P6, MM-1 A/D 0292P6;

Figs. 8A to 8H show the typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, A+B, B+E, and D+E and the KB Nasopharyngeal/HeLa 25 cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1A092K3, MM-1 B 0212K5, MM-1 C 0162K3, MM-1 D 0212K5, MM-1 E 0292K5, MM-1 A/B 0292K3, MM-1 B/E 0292K4, MM-1 D/E 0292K5;

Figs. 9A to 9H show the typical dose response 30 relationship between cocoa procyanidin fractions A, B, C, E, B+D, A+E and D+E and the HCT-116 cell line (fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 C 0192H5, D 0192H5, E 0192H5, MM-1 B&D 0262H2, A/E 0262H3, MM-1 D&E 0262H1;

35 Figs. 10A to 10H show typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, B+D, C+D and A+E and the ACHN renal cell line

(fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 A 092A5, MM-1 B 092A5, MM-1 C 0192A7, MM-1 D 0192A7, M&M1 E 0192A7, MM-1 B&D 0302A6, MM-1 C&D 0302A6, MM-1 A&E 0262A6;

Figs. 11A to 11H show typical dose response
5 relationships between cocoa procyanidin fractions A, B,
C, D, E, A+E, B+E and C+E and the A-549 lung cell line
(fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 A 019258, MM-
1 B 09256, MM-1 C 019259, MM-1 D 019258, MM-1 E 019258,
A/E 026254, MM-1 B&E 030255, MM-1 C&E N6255;

10 Figs. 12A to 12H show typical dose response
relationships between cocoa procyanidin fractions A, B,
C, D, E, B+C, C+D and D+E and the SK-5 melanoma cell line
(fractional survival vs. dose $\mu\text{g/mL}$); MM-1 A 0212S4, MM-1
B 0212S4, MM-1 C 0212S4, MM-1 D 0212S4, MM-1 E N32S1,
15 MM-1 B&C N32S2, MM-1 C&D N32S3, MM-1 D&E N32S3;

Figs. 13A to 13H show typical dose response
relationships between cocoa procyanidin fractions A, B,
C, D, E, B+C, C+E, and D+E and the MCF-7 breast cell line
(fractional survival vs. dose, $\mu\text{g/mL}$); MM-1 A N22M4, MM-1
20 B N22M4, MM-1 C N22M4, MM-1 D N22M3, MM-1 E 0302M2, MM-1
B/C 0302M4, MM-1 C&E N22M3, MM-1 D&E N22M3;

Fig. 14 shows typical dose response
relationships for cocoa procyanidin (particularly
fraction D) and the CCRF-CEM T-cell leukemia cell line
25 (cells/mL vs. days of growth; open circle is control,
darkened circle is 125 μg fraction D, open inverted
triangle is 250 μg fraction D, darkened inverted triangle
is 500 μg fraction D);

Fig. 15A shows a comparison of the XTT and
30 Crystal Violet cytotoxicity assays against MCF-7 p168
breast cancer cells treated with fraction D+E (open
circle is XTT and darkened circle is Crystal Violet);

Fig. 15B shows a typical dose response curve
obtained from MDA MB231 breast cell line treated with
35 varying levels of crude polyphenols obtained from UIT-1
cocoa genotype (absorbance (540nm) vs. Days; open circle

is control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL, open square is 10 μ g/mL; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15C shows a typical dose response curve obtained from PC-3 prostate cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540nm) vs. Days; open circle 10 is control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL and open square is 10 μ g/mL);

Fig. 15D shows a typical dose-response curve obtained from MCF-7 p168 breast cancer cell line treated 15 with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL, open square is 10 μ g/mL, darkened square is 1 μ g/mL; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15E shows a typical dose response curve obtained from Hela cervical cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 25 cocoa genotype (absorbance (540nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250 μ g/mL, darkened inverted triangle is 100 μ g/mL, open square is 10 μ g/mL; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15F shows cytotoxic effects against Hela cervical cancer cell line treated with different cocoa polyphenol fractions (absorbance (540nm) vs. Days; open circle is 100 μ g/mL fractions A-E, darkened circle is 35 100 μ g/mL fractions A-C, open inverted triangle is 100 μ g/mL fractions D&E; absorbance of 2.0 is maximum of plate reader and not representative of cell number);

Fig. 15G shows cytotoxic effects at 100 μ L/mL against SKBR-3 breast cancer cell line treated with different cocoa polyphenol fractions (absorbance (540nm) vs. Days; open circle is fractions A-E, darkened circle 5 is fractions A-C, open inverted triangle is fractions D&E);

Fig. 15H shows typical dose-response relationships between cocoa procyanidin fraction D+E on Hela cells (absorbance (540nm) vs. Days; open circle is 10 control, darkened circle is 100 μ g/mL, open inverted triangle is 75 μ g/mL, darkened inverted triangle is 50 μ g/mL, open square is 25 μ g/mL, darkened square is 10 μ g/mL; absorbance of 2.0 is maximum of plate reader and is not representative of cell number);

15 Fig. 15I shows typical dose-response relationship between cocoa procyanidin fraction D+E on SKBR-3 cells (absorbance (540nm) vs. Days; open circle is control, darkened circle is 100 μ g/mL, open inverted triangle is 75 μ g/mL, darkened inverted triangle is 50 μ g/mL, open square is 25 μ g/mL, darkened square is 10 μ g/mL);

Fig. 15J shows typical dose-response relationships between cocoa procyanidin fraction D+E on Hela cells using the Soft Agar Cloning assay (bar chart; 25 number of colonies vs. control, 1, 10, 50, and 100 μ g/mL);

Fig. 15K shows the growth inhibition of Hela cells when treated with crude polyphenol extracts obtained from eight different cocoa genotypes (% control vs. concentration, μ g/mL; open circle is C-1, darkened circle is C-2, open inverted triangle is C-3, darkened inverted triangle is C-4, open square is C-5, darkened square is C-6, open triangle is C-7, darkened triangle is C-8; C-1 = UF-12: horti race = Trinitario and description is crude extracts of UF-12 (Brazil) cocoa polyphenols 35 (decaffeinated/detheobrominated); C-2 = NA-33: horti race = Forastero and description is crude extracts of NA-33 (Brazil) cocoa polyphenols (decaffeinated/

detheobrominated); C-3 = EEG-48: horti race = Forastero and description is crude extracts of EEG-48 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-4 = unknown: horti race = Forastero and description is crude extracts of unknown (W. African) cocoa polyphenols (decaffeinated/detheobrominated); C-5 = UF-613: horti race = Trinitario and description is crude extracts of UF-613 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-6 = ICS-100: horti race = Trinitario 10 (to Nicaraguan Criollo ancestor) and description is crude extracts of ICS-100 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-7 = ICS-139: horti race = Trinitario (Nicaraguan Criollo ancestor) and description is crude extracts of ICS-139 (Brazil) cocoa 15 polyphenols (decaffeinated/detheobrominated); C-8 = UIT-1: horti race = Trinitario and description is crude extracts of UIT-1 (Malaysia) cocoa polyphenols (decaffeinated/detheobrominated);

Fig. 15L shows the growth inhibition of Hela 20 cells when treated with crude polyphenol extracts obtained from fermented cocoa beans and dried cocoa beans (stages throughout fermentation and sun drying; % control vs. concentration, $\mu\text{g/mL}$; open circle is day zero fraction, darkened circle is day 1 fraction, open 25 inverted triangle is day 2 fraction, darkened inverted triangle is day 3 fraction, open square is day 4 fraction and darkened square is day 9 fraction);

Fig. 15M shows the effect of enzymatically oxidized cocoa procyanidins against Hela cells (dose 30 response for polyphenol oxidase treated crude cocoa polyphenol; % control vs. concentration, $\mu\text{g/mL}$; darkened square is crude UIT-1 (with caffeine and theobromine), open circle crude UIT-1 (without caffeine and theobromine) and darkened circle is crude UIT-1 35 (polyphenol oxidase catalyzed);

Fig. 15N shows a representative semi-preparative reverse phase HPLC separation for combined cocoa procyanidin fractions D and E;

Fig. 15O shows a representative normal phase semi-preparative HPLC separation of a crude cocoa polyphenol extract;

Fig. 16 shows typical Rancimat Oxidation curves for cocoa procyanidin extract and fractions in comparison to the synthetic antioxidants BHA and BHT (arbitrary units vs. time; dotted line and cross (+) is BHA and BHT; * is D-E; x is crude; open square is A-C; and open diamond is control);

Fig. 17 shows a typical Agarose Gel indicating inhibition of topoisomerase II catalyzed decatenation of kinetoplast DNA by cocoa procyanidin fractions (Lane 1 contains 0.5 μ g of marker (M) monomer-length kinetoplast DNA circles; Lanes 2 and 20 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 4% DMSO, but in the absence of any cocoa procyanidins. 20 (Control -C); Lanes 3 and 4 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.5 and 5.0 μ g/mL cocoa procyanidin fraction A; Lanes 5 and 6 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.5 and 5.0 μ g/mL 25 cocoa procyanidin fraction B; Lanes 7, 8, 9, 13, 14 and 15 are replicates of kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.05, 0.5 and 5.0 μ g/mL cocoa procyanidin fraction D; Lanes 10, 11, 12, 16, 17 and 18 are replicates of kinetoplast DNA that was 30 incubated with Topoisomerase II in the presence of 0.05, 0.5, and 5.0 μ g/mL cocoa procyanidin fraction E; Lane 19 is a replicate of kinetoplast DNA that was incubated with Topoisomerase II in the presence of 5.0 μ g/mL cocoa procyanidin fraction E);

35 Fig. 18 shows dose response relationships of cocoa procyanidin fraction D against DNA repair competent and deficient cell lines (fractional survival vs. μ g/mL;

left side xrs-6 DNA Deficient Repair Cell Line, MM-1 D D282X1; right side BR1 Competent DNA Repair Cell Line, MM-1 D D282B1);

Fig. 19 shows the dose-response curves for 5 Adriamycin resistant MCF-7 cells in comparison to a MCF-7 p168 parental cell line when treated with cocoa fraction D+E (% control vs. concentration, $\mu\text{g/mL}$; open circle is MCF-7 p168; darkened circle is MCF-7 ADR);

Figs. 20A and B show the dose-response effects 10 on Hela and SKBR-3 cells when treated at 100 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$ levels of twelve fractions prepared by Normal phase semi-preparative HPLC (bar chart, % control vs. control and fractions 1-12);

Fig. 21 shows a normal phase HPLC separation of 15 crude, enriched and purified pentamers from cocoa extract;

Figs. 22A, B and C show MALDI-TOF/MS of pentamer enriched procyanidins, and of Fractions A-C and of Fractions D-E, respectively;

Fig. 23A shows an elution profile of oligomeric 20 procyanidins purified by modified semi-preparative HPLC;

Fig. 23B shows an elution profile of a trimer procyanidin by modified semi-preparative HPLC;

Figs. 24A-D each show energy minimized 25 structures of all (4-8) linked pentamers based on the structure of epicatechin;

Fig. 25A shows relative fluorescence of epicatechin upon thiolysis with benzylmercapten;

Fig. 25B shows relative fluorescence of 30 catechin upon thiolysis with benzylmercapten;

Fig. 25C shows relative fluorescence of dimers (B2 and B5) upon thiolysis with benzylmercapten;

Fig. 26A shows relative fluorescence of dimer upon thiolysis;

35 Fig. 26B shows relative fluorescence of B5 dimer upon thiolysis of dimer and subsequent desulphurization;

Fig. 27A shows the relative tumor volume during treatment of MDA MB 231 nude mouse model treated with pentamer;

Fig. 27B shows the relative survival curve of 5 pentamer treated MDA 231 nude mouse model;

Fig. 28 shows the elution profile from halogen-free analytical separation of acetone extract of procyanidins from cocoa extract;

Fig. 29 shows the effect of pore size of 10 stationary phase for normal phase HPLC separation of procyanidins;

Fig. 30A shows the substrate utilization during fermentation of cocoa beans;

Fig. 30B shows the metabolite production during 15 fermentation;

Fig. 30C shows the plate counts during fermentation of cocoa beans;

Fig. 30D shows the relative concentrations of each component in fermented solutions of cocoa beans;

20 Fig. 31 shows the acetylcholine-induced relaxation of NO-related phenylephrine-precontracted rat aorta;

Fig. 32 shows the blood glucose tolerance profiles from various test mixtures;

25 Figs. 33A-B show the effects of indomethacin on COX-1 and COX-2 activities;

Figs. 34A-B show the correlation between the degree of polymerization and IC₅₀ vs. COX-1/COX-2 (μ M);

30 Fig. 35 shows the correlation between the effects of compounds on COX-1 and COX-2 activities expressed as μ M;

Figs. 36A-V show the IC₅₀ values (μ M) of samples containing procyanidins with COX-1/COX-2;

35 Fig. 37 shows the purification scheme for the isolation of procyanidins from cocoa;

Fig. 38A to 38P shows the preferred structures of the pentamer;

Figs. 39A-AA show a library of stereoisomers of pentamers;

Figs. 40A-B show 70 minute gradients for normal phase HPLC separation of procyanidins, detected by UV and fluorescence, respectively;

Figs. 41A-B show 30 minute gradients for normal phase HPLC separation of procyanidins, detected by UV and fluorescence, respectively;

Fig. 42 shows a preparation normal phase HPLC separation of procyanidins;

Figs. 43A-G show CD (circular dichroism) spectra of procyanidin dimers, trimers, tetramers, pentamers, hexamers, heptamers and octamers, respectively;

Fig. 44A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for epicatechin;

Figs. 44B-F show the APT, COSY, XHCORR, ^1H and ^{13}C NMR spectra for epicatechin;

Fig. 45A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for catechin;

Figs. 45B-E show the ^1H , APT, XHCORR and COSY NMR spectra for catechin;

Fig. 46A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for B2 dimer;

Figs. 46B-G show the ^{13}C , APT, ^1H , HMQC, COSY and HOHAHA NMR spectra for the B2 dimer;

Fig. 47A shows the structure and $^1\text{H}/^{13}\text{C}$ NMR data for B5 dimer;

Figs. 47B-G show the ^1H , ^{13}C , APT, COSY, HMQC and HOHAHA NMR spectra for B5 dimer;

Figs. 48A-D show the ^1H , COSY, HMQC and HOHAHA NMR spectra for epicatechin/catechin trimer;

Figs. 49A-D show the ^1H , COSY, HMQC and HOHAHA NMR spectra for epicatechin trimer;

Figs. 50A and B show the effects of cocoa procyanidin fraction A and C, respectively, on blood pressure; blood pressure levels decreased by 21.43%

within 1 minute after administration of fraction A, and returned to normal after 15 minutes, while blood pressure decreased by 50.5% within 1 minute after administration of fraction C, and returned to normal after 5 minutes;

5 Fig. 51 shows the effect of cocoa procyanidin fractions on arterial blood pressure in anesthetized guinea pigs;

Fig. 52 shows the effect of L-NMMA on the alterations of arterial blood pressure in anesthetized 10 guinea pigs induced by cocoa procyanidin fraction C;

Fig. 53 shows the effect of bradykinin on NO production by HUVEC;

Fig. 54 shows the effect of cocoa procyanidin fractions on macrophage NO production by HUVEC;

15 Fig. 55 shows the effect of cocoa procyanidin fractions on macrophage NO production;

Fig. 56 shows the effect of cocoa procyanidin fraction on LPS induced and gamma-Interferon primed macrophages.

20 Fig. 57 shows a micellar electrokinetic capillary chromatographic separation of cocoa procyanidin oligomers;

Fig. 58 A-F show MALDI-TOF mass spectra for Cu⁺²-, Zn⁺²-, Fe⁺²-, Fe⁺³-, Ca⁺²-, and Mg⁺²- ions, 25 respectively, complexed to a trimer;

Fig. 59 shows a MALDI-TOF mass spectrum of cocoa procyanidin oligomers (tetramers to octadecamers);

Fig. 60 shows the dose-response relationship of cocoa procyanidin oligomers and the feline FeA 30 lymphoblastoid cell line producing leukemia virus;

Fig. 61 shows the dose-response relationship of cocoa procyanidin oligomers and the feline CRFK normal kidney cell line;

Fig. 62 shows the dose-response relationship of 35 cocoa procyanidin oligomers and the canine MDCK normal kidney line;

Fig. 63 shows the dose-response relationship between cocoa procyanidin oligomers and the canine GH normal kidney cell line;

Fig. 64 shows time-temperature effects on 5 hexamer hydrolysis; and

Fig. 65 shows time-temperature effects on trimer formation.

DETAILED DESCRIPTION

COMPOUNDS OF THE INVENTION

10 As discussed above, it has now been surprisingly found that cocoa extracts or compounds derived therefrom exhibit anti-cancer, anti-tumor or antineoplastic activity, antioxidant activity, inhibit DNA topoisomerase II enzyme and oxidative damage to DNA, 15 and have antimicrobial, cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase, apoptosis, platelet aggregation and blood or *in vivo* glucose, modulating activities, as well as efficacy as a non-steroidal antiinflammatory agent.

20 The extracts, compounds or combination of compounds derived therefrom are generally prepared by reducing cocoa beans to a powder, defatting the powder, and extracting and purifying the active compound(s) from the defatted powder. The powder can be prepared by 25 freeze-drying the cocoa beans and pulp, depulping and dehulling the freeze-dried cocoa beans and grinding the dehulled beans. The extraction of active compound(s) can be by solvent extraction techniques. The extracts comprising the active compounds can be purified, e.g., to 30 be substantially pure, for instance, by gel permeation chromatography or by preparative High Performance Liquid Chromatography (HPLC) techniques or by a combination of such techniques.

With reference to the isolation and 35 purification of the compounds of the invention derived from cocoa, it will be understood that any species of *Theobroma*, *Herrania* or inter- and intra-species crosses

thereof may be employed. In this regard, reference is made to Schultes, "Synopsis of *Herrania*," Journal of the Arnold Arboretum, Vol. XXXIX, pp. 217 to 278, plus plates I to XVII (1985), Cuatrecasas, "Cocoa and Its Allies, A 5 Taxonomic Revision of the Genus *Theobroma*," Bulletin of the United States National Museum, Vol. 35, part 6, pp. 379 to 613, plus plates 1 to 11 (Smithsonian Institution, 1964), and Addison, et al., "Observations on the Species of the Genus *Theobroma* Which Occurs in the Amazon," Bol. 10 Tech. Inst. Agronomico de Nortes, 25(3) (1951).

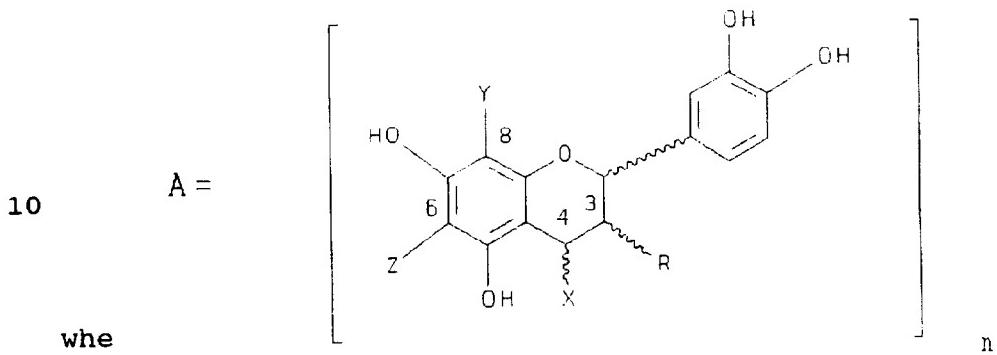
Additionally, Example 25 lists the heretofore never reported concentrations of the inventive compounds found in *Theobroma* and *Herrania* species and their inter- and intra-species crosses; and Example 25 also describes 15 methods of modulating the amounts of the inventive compounds which may be obtained from cocoa by manipulating cocoa fermentation conditions.

An outline of the purification protocol utilized in the isolation of substantially pure 20 procyanidins is shown in Fig. 37. Steps 1 and 2 of the purification scheme are described in Examples 1 and 2; steps 3 and 4 are described in Examples 3, 13 and 23; step 5 is described in Examples 4 and 14; and step 6 is described in Examples 4, 14 and 16. The skilled artisan 25 would appreciate and envision modifications in the purification scheme outlined in Figure 37 to obtain the active compounds without departing from the spirit or scope thereof and without undue experimentation.

The extracts, compounds and combinations of 30 compounds derived therefrom having activity, without wishing to necessarily be bound by any particular theory, have been identified as cocoa polyphenol(s), such as procyanidins. These cocoa procyanidins have significant anti-cancer, anti-tumor or antineoplastic activity; 35 antioxidant activity; inhibit DNA topoisomerase II enzyme and oxidative damage to DNA; possess antimicrobial activity; have the ability to modulate cyclo-oxygenase

and/or lipoxygenase, NO or NO-synthase, apoptosis, platelet aggregation and blood or *in vivo* glucose, and have efficacy as non-steroidal antiinflammatory agents.

The present invention provides a compound of 5 the formula:

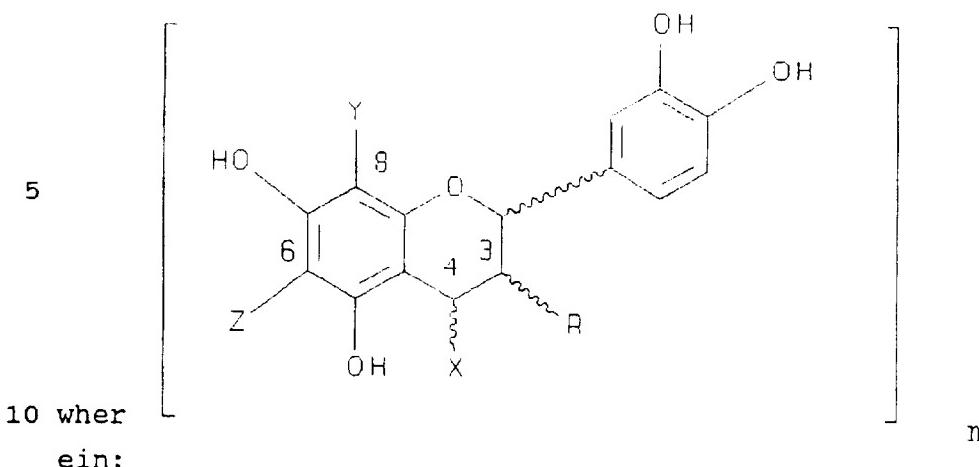


n is an integer from 2 to 18, e.g., 3 to 12, such that there is a first monomeric unit A, and a plurality of other monomeric units;

20 R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

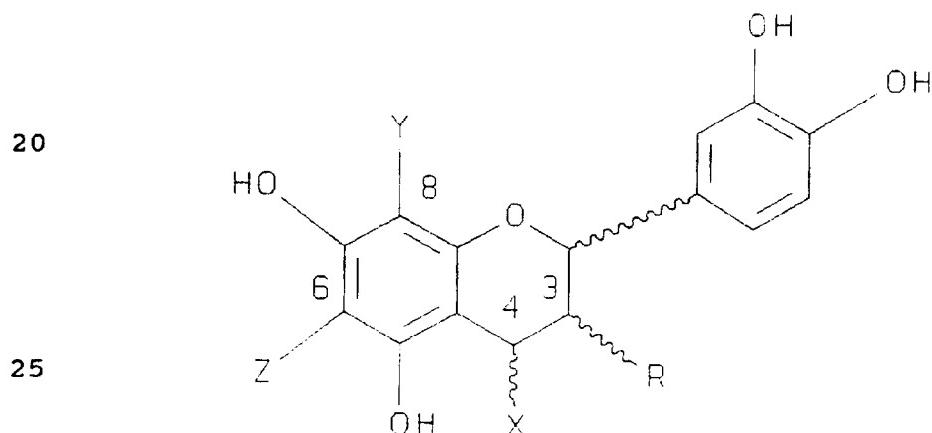
position 4 is alpha or beta stereochemistry;
 X, Y and Z represent positions for bonding between monomeric units, with the provisos that as to the first monomeric unit, bonding of another monomeric unit thereto is at position 4 and Y = Z = hydrogen, and, that when not for bonding monomeric units, X, Y and Z are hydrogen, or Z, Y are sugar and X is hydrogen, or X is alpha or beta sugar and Z, Y are hydrogen, or combinations thereof. The compound can have n as 5 to 30 12, and certain preferred compounds have n as 5. The sugar can be selected from the group consisting of glucose, galactose, xylose, rhamnose, and arabinose. The sugar of any or all of R, X, Y and Z can optionally be substituted with a phenolic moiety via an ester bond.

35 Thus, the invention can provide a compound of the formula:



n is an integer from 2 to 18, e.g., 3 to 12, advantageously 5 to 12, and preferably n is 5, such that there is a first monomeric unit A,

15



and a plurality of other monomeric units of A;
R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-
30 (β)-O-sugar;

position 4 is alpha or beta stereochemistry;
X, Y and Z represent positions for bonding between monomeric units, with the provisos that as to the first monomeric unit, bonding of another monomeric unit thereto is at position 4 and Y = Z = hydrogen, and, that when not for bonding monomeric units, X, Y and Z are hydrogen or Z, Y are sugar and X is hydrogen, or X is

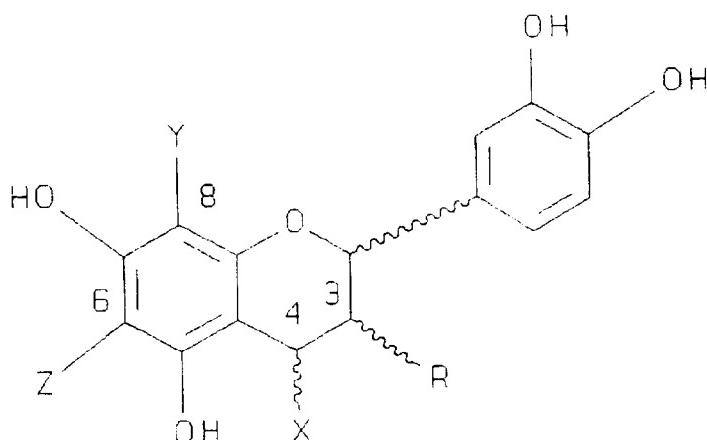
alpha or beta sugar and Z and Y are hydrogen, or combinations thereof; and

said sugar is optionally substituted with a phenolic moiety via an ester bond.

5 Accordingly, the present invention provides a polymeric compound of the formula A_n , wherein A is a monomer having the formula:

10

15



wherein

20 n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and at least one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

25 bonding between adjacent monomers takes place at positions 4, 6 or 8;

a bond of an additional monomeric unit in position 4 has α or β stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto (i.e., the bonding of the monomeric unit adjacent the terminal monomeric unit) is at position 4 and

35 optionally, Y = Z = hydrogen;

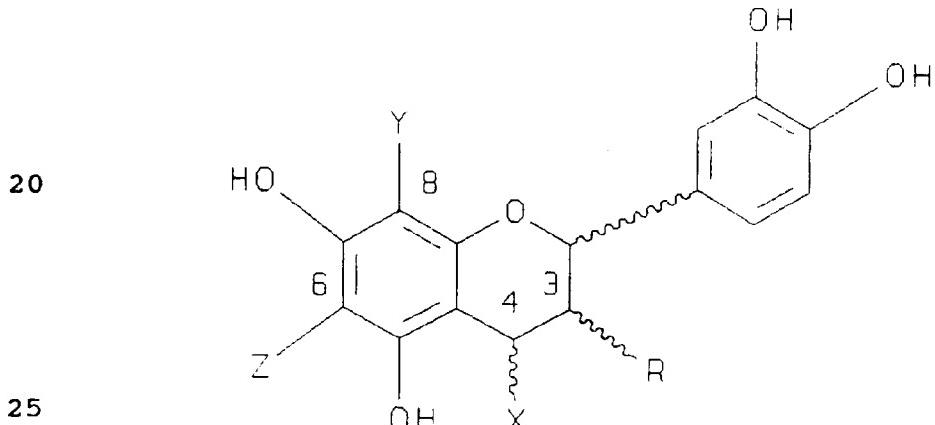
the sugar is optionally substituted with a phenolic moiety at any position, for instance via an

ester bond, and pharmaceutically acceptable salts or derivatives thereof (including oxidation products).

In preferred embodiments, n can be 3 to 18, 2 to 18, 3 to 12, e.g., 5 to 12; and, advantageously, n is 5. The sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose. The sugar of any or all of R, X, Y and Z can optionally be substituted at any position with a phenolic moiety via an ester bond. The phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

Additionally, the present invention provides a polymeric compound of the formula A_n, wherein A is a monomer having the formula:

15



wherein

n is an integer from 2 to 18, e.g., 3 to 18, advantageously 3 to 12, e.g., 5 to 12, preferably n is 5;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-30 (β)-O-sugar;

adjacent monomers bind at position 4 by (4-6) or (4-8);

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent 35 monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent

monomer is at position 4 and optionally, Y = Z = hydrogen;

a bond at position 4 has α or β stereochemistry;

5 the sugar is optionally substituted with a phenolic moiety at any position, for instance, via an ester bond,

and pharmaceutically acceptable salts or derivatives thereof (including oxidation products).

10 With regard to the recitation of "at least one terminal monomeric unit A", it will be understood that the inventive compounds have two terminal monomeric units, and that the two terminal monomeric unit A may be the same or different. Additionally, it will be
15 understood that the recitation of "at least one terminal monomeric unit A" includes embodiments wherein the terminal monomeric unit A is referred to as a "first monomeric unit", with the recitation of "first monomeric unit" relating to that monomer to which other monomeric
20 units are added, resulting in a polymeric compound of the formula A_n . Moreover, with regard to the at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

25 As to the recitation of the term "combinations thereof", it will be understood that one or more of the inventive compounds may be used simultaneously, e.g., administered to a subject in need of treatment in a formulation comprising one or more inventive compounds.

30 The inventive compounds or combinations thereof display the utilities noted above for cocoa extracts; and throughout the disclosure, the term "cocoa extract" may be substituted by compounds of the invention or combinations thereof, such that it will be understood
35 that the inventive compounds or combinations thereof can be cocoa extracts.

The term "oligomer", as used herein, refers to any compounds or combinations thereof of the formula presented above, wherein n is 2 through 18. When n is 2, the oligomer is termed a "dimer"; when n is 3, the 5 oligomer is termed a "trimer"; when n is 4, the oligomer is termed a "tetramer"; when n is 5, the oligomer is termed a "pentamer"; and similar recitations may be designated for oligomers having n up to and including 18, such that when n is 18, the oligomer is termed an 10 "octadecamer".

The inventive compounds or combinations thereof can be isolated, e.g., from a natural source such as any species of *Theobroma*, *Herrania* or inter- or intra-species crosses thereof; or, the inventive compounds or 15 combinations thereof can be purified, e.g., compounds or combinations thereof can be substantially pure; for instance, purified to apparent homogeneity. Purity is a relative concept, and the numerous Examples demonstrate isolation of inventive compounds or combinations thereof, 20 as well as purification thereof, such that by methods exemplified a skilled artisan can obtain a substantially pure inventive compound or combination thereof, or purify them to apparent homogeneity (e.g., purity by separate, distinct chromatographic peak). Considering the Examples 25 (e.g., Example 37), a substantially pure compound or combination of compounds is at least about 40% pure, e.g., at least about 50% pure, advantageously at least about 60% pure, e.g., at least about 70% pure, more advantageously at least about 75-80% pure, preferably, at 30 least about 90% pure, more preferably greater than 90% pure, e.g., at least 90-95% pure, or even purer, such as greater than 95% pure, e.g., 95-98% pure.

Further, examples of the monomeric units comprising the oligomers used herein are (+)-catechin and

35

(-)-epicatechin, abbreviated C and EC, respectively. The linkages between adjacent monomers are from position 4 to

position 6 or position 4 to position 8; and this linkage between position 4 of a monomer and position 6 and 8 of the adjacent monomeric units is designated herein as (4 \rightarrow 6) or (4 \rightarrow 8). There are four possible stereochemical linkages between position 4 of a monomer and position 6 and 8 of the adjacent monomer; and the stereochemical linkages between monomeric units is designated herein as (4 α \rightarrow 6) or (4 β \rightarrow 6) or (4 α \rightarrow 8) or (4 β \rightarrow 8). When C is linked to another C or EC, the linkages are designated herein as 10 (4 α \rightarrow 6) or (4 α \rightarrow 8). When EC is linked to another C or EC, the linkages are designated herein as (4 β \rightarrow 6) or (4 β \rightarrow 8).

Examples of compounds eliciting the activities cited above include dimers, EC-(4 β \rightarrow 8)-EC and EC-(4 β \rightarrow 6)-EC, wherein EC-(4 β \rightarrow 8)-EC is preferred; trimers [EC-15 (4 β \rightarrow 8)]₂-EC, [EC-(4 β \rightarrow 8)]₂-C and [EC-(4 β \rightarrow 6)]₂-EC, wherein [EC-(4 β \rightarrow 8)]₂-EC is preferred; tetramers [EC-(4 β \rightarrow 8)]₃-EC, [EC-(4 β \rightarrow 8)]₃-C and [EC-(4 β \rightarrow 8)]₂-EC-(4 β \rightarrow 6)-C, wherein [EC-(4 β \rightarrow 8)]₃-EC is preferred; and pentamers [EC-(4 β \rightarrow 8)]₄-EC, [EC-(4 β \rightarrow 8)]₃-EC-(4 β \rightarrow 6)-EC, [EC-(4 β \rightarrow 8)]₃-EC-(4 β \rightarrow 8)-C and 20 [EC-(4 β \rightarrow 8)]₃-EC-(4 β \rightarrow 6)-C, wherein the 3-position of the pentamer terminal monomeric unit is optionally derivatized with a gallate or β -D-glucose; [EC-(4 β \rightarrow 8)]₄-EC is preferred.

Additionally, compounds which elicit the 25 activities cited above also include hexamers to dodecamers, examples of which are listed below:

A hexamer, wherein one monomer (C or EC) having linkages to another monomer (4 β \rightarrow 8) or (4 β \rightarrow 6) for EC linked to another EC or C, and (4 α \rightarrow 8) or (4 α \rightarrow 6) for C 30 linked to another C or EC; followed by a (4 β \rightarrow 8) linkage to a pentamer compound listed above, e.g., [EC-(4 β \rightarrow 8)]₅-EC, [EC-(4 β \rightarrow 8)]₄-EC-(4 β \rightarrow 6)-EC, [EC-(4 β \rightarrow 8)]₄-EC-(4 β \rightarrow 8)-C, and [EC-(4 β \rightarrow 8)]₄-EC-(4 β \rightarrow 6)-C, wherein the 3-position of the hexamer terminal monomeric unit is optionally 35 derivatized with a gallate or a β -D-glucose; in a preferred embodiment, the hexamer is [EC-(4 β \rightarrow 8)]₅-EC;

A heptamer, wherein any combination of two monomers (C and/or EC) having linkages to one another (4 β -8) or (4 β -6) for EC linked to another EC or C, and (4 α -8) or (4 α -6) for C linked to another C or EC; 5 followed by a (4 β -8) linkage to a pentamer compound listed above, e.g., [EC-(4 β -8)]₆-EC, [EC-(4 β -8)]₅-EC-(4 β -6)-EC, [EC-(4 β -8)]₅-EC-(4 β -8)-C, and [EC-(4 β -8)]₅-EC-(4 β -6)-C, wherein the 3-position of the heptamer terminal monomeric unit is optionally derivatized with a gallate 10 or a β -D-glucose; in a preferred embodiment, the heptamer is [EC-(4 β -8)]₆-EC;

An octamer, wherein any combination of three monomers (C and/or EC) having linkages to one another (4 β -8) or (4 β -6) for EC linked to another EC or C, and 15 (4 α -8) or (4 α -6) for C linked to another C or EC; followed by a (4 β -8) linkage to a pentamer compound listed above, e.g., [EC-(4 β -8)]₇-EC, [EC-(4 β -8)]₆-EC-(4 β -6)-EC, [EC-(4 β -8)]₆-EC-(4 β -8)-C, and [EC-(4 β -8)]₆-EC-(4 β -6)-C, wherein the 3-position of the octamer terminal 20 monomeric unit is optionally derivatized with a gallate or a β -D-glucose; in a preferred embodiment, the octamer is [EC-(4 β -8)]₇-EC;

A nonamer, wherein any combination of four monomers (C and/or EC) having linkages to one another 25 (4 β -8) or (4 β -6) for EC linked to another EC or C, and (4 α -8) or (4 α -6) for C linked to another C or EC; followed by a (4 β -8) linkage to a pentamer compound listed above, e.g., [EC-(4 β -8)]₈-EC, [EC-(4 β -8)]₇-EC-(4 β -6)-EC, [EC-(4 β -8)]₇-EC-(4 β -8)-C, and [EC-(4 β -8)]₇-EC-30 (4 β -6)-C, wherein the 3-position of the nonamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose; in a preferred embodiment, the nonamer is [EC-(4 β -8)]₈-EC;

A decamer, wherein any combination of five 35 monomers (C and/or EC) having linkages to one another (4 β -8) or (4 β -6) for EC linked to another EC or C, and (4 α -8) or (4 α -6) for C linked to another C or EC;

followed by a ($4\beta\rightarrow 8$) linkage to a pentamer compound listed above, e.g., [EC-($4\beta\rightarrow 8$)]₉-EC, [EC-($4\beta\rightarrow 8$)]₈-EC-($4\beta\rightarrow 6$)-EC, [EC-($4\beta\rightarrow 8$)]₈-EC-($4\beta\rightarrow 8$)-C, and [EC-($4\beta\rightarrow 8$)]₈-EC-($4\beta\rightarrow 6$)-C, wherein the 3-position of the decamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose; in a preferred embodiment, the decamer is [EC-($4\beta\rightarrow 8$)]₉-EC;

An undecamer, wherein any combination of six monomers (C and/or EC) having linkages to one another 10 ($4\beta\rightarrow 8$) or ($4\beta\rightarrow 6$) for EC linked to another EC or C, and ($4\alpha\rightarrow 8$) or ($4\alpha\rightarrow 6$) for C linked to another C or EC; followed by a ($4\beta\rightarrow 8$) linkage to a pentamer compound listed above, e.g., [EC-($4\beta\rightarrow 8$)]₁₀-EC, [EC-($4\beta\rightarrow 8$)]₉-EC-($4\beta\rightarrow 6$)-EC, [EC-($4\beta\rightarrow 8$)]₉-EC-($4\beta\rightarrow 8$)-C, and [EC-($4\beta\rightarrow 8$)]₉-EC-15 ($4\beta\rightarrow 6$)-C, wherein the 3-position of the undecamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose; in a preferred embodiment, the undecamer is [EC-($4\beta\rightarrow 8$)]₁₀-EC; and

A dodecamer, wherein any combination of seven 20 monomers (C and/or EC) having linkages to one another ($4\beta\rightarrow 8$) or ($4\beta\rightarrow 6$) for EC linked to another EC or C, and ($4\alpha\rightarrow 8$) or ($4\alpha\rightarrow 6$) for C linked to another C or EC; followed by a ($4\beta\rightarrow 8$) linkage to a pentamer compound listed above, e.g., [EC-($4\beta\rightarrow 8$)]₁₁-EC, [EC-($4\beta\rightarrow 8$)]₁₀-EC-25 ($4\beta\rightarrow 6$)-EC, [EC-($4\beta\rightarrow 8$)]₁₀-EC-($4\beta\rightarrow 8$)-C, and [EC-($4\beta\rightarrow 8$)]₁₀-EC-($4\beta\rightarrow 6$)-C, wherein the 3-position of the dodecamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose; in a preferred embodiment, the dodecamer is [EC-($4\beta\rightarrow 8$)]₁₁-EC.

30 It will be understood from the detailed description that the aforementioned list is exemplary and provided as an illustrative source of several non-limiting examples of compounds of the invention, which is by no means an exhaustive list of the inventive compounds encompassed by the present invention.

Examples 3A, 3B, 4, 14, 23, 24, 30 and 34 describe methods to separate the compounds of the

invention. Examples 13, 14A-D and 16 describe methods to purify the compounds of the invention. Examples 5, 15, 18, 19, 20 and 29 describe methods to identify compounds of the invention. Figures 38A-P and 39A-AA illustrate a 5 stereochemical library for representative pentamers of the invention. Example 17 describes a method to molecularly model the compounds of the invention. Example 36 provides evidence for higher oligomers in cocoa, wherein n is 13 to 18.

10 Furthermore, while the invention is described with respect to cocoa extracts preferably comprising cocoa procyanidins, from this disclosure the skilled organic chemist will appreciate and envision synthetic routes to obtain and/or prepare the active compounds (see 15 e.g., Example 11). Accordingly, the invention comprehends synthetic cocoa polyphenols or procyanidins or their derivatives and/or their synthetic precursors which include, but are not limited to glycosides, gallates, esters, etc. and the like. That is, the 20 inventive compounds can be prepared from isolation from cocoa or from any species within the *Theobroma* or *Herrania* genera, as well as from synthetic routes; and derivatives and synthetic precursors of the inventive compounds such as glycosides, gallates, esters, etc. are 25 included in the inventive compounds. Derivatives can also include compounds of the above formulae wherein a sugar or gallate moiety is on the terminal monomer at positions Y or Z, or a substituted sugar or gallate moiety is on the terminal monomer at Y or Z.

30 For example, Example 8, Method C describes the use of cocoa enzymes to oxidatively modify the compounds of the invention or combinations thereof to elicit improved cytotoxicity (see Fig. 15M) against certain cancer cell lines. The invention includes the ability to 35 enzymatically modify (e.g., cleavage or addition of a chemically significant moiety) the compounds of the invention, e.g., enzymatically with polyphenol oxidase,

peroxidase, catalase combinations, and/or enzymes such as hydrolases, esterases, reductases, transferases, and the like and in any combination, taking into account kinetic and thermodynamic factors (see also Example 41 regarding 5 hydrolysis).

With regard to the synthesis of the inventive compounds, the skilled artisan will be able to envision additional routes of synthesis, based on this disclosure and the knowledge in the art, without undue 10 experimentation. For example, based upon a careful retrosynthetic analysis of the polymeric compounds, as well as the monomers. For instance, given the phenolic character of the inventive compounds, the skilled artisan can utilize various methods of selective 15 protection/deprotection, coupled with organometallic additions, phenolic couplings and photochemical reactions, e.g., in a convergent, linear or biomimetic approach, or combinations thereof, together with standard reactions known to those well-versed in the art of 20 synthetic organic chemistry, as additional synthetic methods for preparing the inventive compounds, without undue experimentation. In this regard, reference is made to W. Carruthers, Some Modern Methods of Organic Synthesis, 3rd ed., Cambridge University Press, 1986, and 25 J. March, Advanced Organic Chemistry, 3rd ed., John Wiley & Sons, 1985, van Rensburg et al., Chem. Comm., 24: 2705-2706 (Dec. 21, 1996), Ballenegger et al., (Zyma SA) European Patent 0096 007 B1, and documents in the References section below, all of which are hereby 30 incorporated herein by reference.

UTILITIES OF COMPOUNDS OF THE INVENTION

With regard to the inventive compounds, it has been surprisingly found that the inventive compounds have discrete activities, and as such, the inventive compounds 35 have broad applicability to the treatment of a variety of disease conditions, discussed hereinbelow.

COX/LOX-ASSOCIATED UTILITIES

Atherosclerosis, the most prevalent of cardiovascular diseases, is the principle cause of heart attack, stroke and vascular circulation problems.

Atherosclerosis is a complex disease which involves many 5 cell types, biochemical events and molecular factors. There are several aspects of this disease, its disease states and disease progression which are distinguished by the interdependent consequences of Low Density Lipoprotein (LDL) oxidation, cyclo-oxygenase 10 (COX)/lipoxygenase (LOX) biochemistry and Nitric Oxide (NO) biochemistry.

Clinical studies have firmly established that the elevated plasma concentrations of LDL are associated with accelerated atherogenesis. The cholesterol that 15 accumulates in atherosclerotic lesions originate primarily in plasma lipoproteins, including LDL. The oxidation of LDL is a critical event in the initiation of atheroma formation and is associated with the enhanced production of superoxide anion radical ($O_2\bullet-$). Oxidation 20 of LDL by $O_2\bullet-$ or other reactive species (e.g., $\bullet OH$, $ONOO\bullet-$, lipid peroxy radical, copper ion, and iron based proteins) reduces the affinity of LDL for uptake in cells via receptor mediated endocytosis. Oxidatively modified LDLs are then rapidly taken up by macrophages which 25 subsequently transform into cells closely resembling the "foam cells" observed in early atherosclerotic lesions.

Oxidized lipoproteins can also promote vascular injury through the formation of lipid hydroperoxides within the LDL particle. This event initiates radical 30 chain oxidation reactions of unsaturated LDL lipids, thus producing more oxidized LDL for macrophage incorporation.

The collective accumulation of foam cells engorged with oxidized LDL from these processes results in early "fatty streak" lesions, which eventually 35 progress to the more advanced complex lesions of atherosclerosis leading to coronary disease.

As discussed generally by Jean Marx at page 320 of Science, Vol. 265 (July 15, 1994), each year about 330,000 patients in the United States undergo coronary and/or peripheral angioplasty, a procedure designed to 5 open up blood vessels, e.g., coronary arteries, clogged by dangerous atherosclerotic plaques (atherosclerosis) and thereby restore normal blood flow. For a majority of these patients, the operation works as intended. Nearly 33% of these patients (and maybe more by some accounts), 10 however, develop restenosis, wherein the treated arteries become quickly clogged again. These patients are no better off, and sometimes worse off, than they were before angioplasty. Excessive proliferation of smooth muscle cells (SMCs) in blood vessel walls contributes to 15 restenosis. Increased accumulation of oxidized LDL within lesion SMCs might contribute to an atherogenic-related process like restenosis. Zhou et al., "Association Between Prior Cytomegalovirus Infection And The Risk Of Restenosis After Coronary Atherectomy," 20 August 29, 1996, New England Journal of Medicine, 335:624-630, and documents cited therein, all incorporated herein by reference. Accordingly, utility of the present invention with respect to atherosclerosis can apply to restenosis.

With regard to the inhibition by the inventive 25 compounds of cyclooxygenases (COX; prostaglandin endoperoxide synthase), it is known that cyclooxygenases are central enzymes in the production of prostaglandins and other arachidonic acid metabolites (i.e., 30 eicosanoids) involved in many physiological processes. COX-1 is a constitutive enzyme expressed in many tissues, including platelets, whereas COX-2, a second isoform of the enzyme, is inducible by various cytokines, hormones and tumor promoters. COX 1 produces thromboxane A₂, 35 which is involved in platelet aggregation, which in turn is involved in the progression of atherosclerosis. Its

inhibition is the basis for the prophylactic effects on cardiovascular disease.

The activity of COX-1 and COX-2 is inhibited by aspirin and other nonsteroidal antiinflammatory drugs 5 (NSAIDs), and the gastric side effects of NSAIDs are believed to be associated with the inhibition of COX-1. Moreover, it has been found that patients taking NSAIDs on a regular basis have a 40 to 50% lower risk of contracting colorectal cancer when compared to persons 10 not being administered these type of medications; and COX-2 mRNA levels are markedly increased in 86% of human colorectal adenocarcinomas.

One significant property of COX-2 expressing cell lines is the enhanced expression of genes which 15 participate in the modulation of apoptosis, i.e., programmed cell death. Several NSAIDs have been implicated in increased cell death and the induction of apoptosis in chicken embryo fibroblasts.

Cellular lipoxygenases are also involved in the 20 oxidative modification of LDL through the peroxidation of unsaturated lipids. The generation of lipid peroxy radicals contributes to the further radical chain oxidation of unsaturated LDL lipids, producing more oxidized LDL for macrophage incorporation.

25 It has been surprisingly found that the inventive compounds have utility in the treatment of diseases associated with COX/LOX. In Example 28, COX was inhibited by individual inventive compounds at concentrations similar to a known NSAID, indomethacin.

30 For COX inhibition, the inventive compounds are oligomers, where n is 2 to 18. In a preferred embodiment, the inventive compounds are oligomers where n is 2 to 10, and more preferably, the inventive compounds are oligomers where n is 2 to 5.

35 Examples of compounds eliciting the inhibitory activity with respect to COX/LOX cited above include

dimers, trimers, tetramers and pentamers, discussed above.

Hence, given the significant inhibitory potency of the inventive compounds on COX-2, coupled with the 5 cytotoxic effects on a putative COX-2 expression colon cancer cell line, the inventive compounds possess apoptotic activity as inhibitors of the multistep progression leading to carcinomas, as well as activity as members of the NSAID family of medications possessing a 10 broad spectrum of prophylactic activities (see, e.g., Example 8, Figs. 9D to 9H).

Further, prostaglandins, the penultimate products of the COX catalyzed conversion of arachidonic acid to prostaglandin H₂, are involved in inflammation, 15 pain, fever, fetal development, labor and platelet aggregation. Therefore, the inventive compounds are efficacious for the same conditions as NSAIDs, e.g., against cardiovascular disease, and stroke, etc. (indeed, the inhibition of platelet COX-1, which reduces 20 thromboxane A₂ production, is the basis for the prophylactic effects of aspirin on cardiovascular disease).

Inflammation is the response of living tissues to injury. It involves a complex series of enzyme 25 activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair.

Inflammation is activated by phospholipase A₂, which liberates arachidonic acid, the substrate for COX and LOX enzymes. COX converts arachidonic acid to the 30 prostaglandin PGE₂, the major eicosanoid detected in inflammatory conditions ranging from acute edema to chronic arthritis. Its inhibition by NSAIDs is a mainstay for treatment.

Arthritis is one of the rheumatic diseases 35 which encompass a wide range of diseases and pathological processes, most of which affect joint tissue. The basic structure affected by these diseases is the connective

tissue which includes synovial membranes, cartilage, bone, tendons, ligaments, and interstitial tissues. Temporary connective tissue syndromes include sprains and strains, tendonitis, and tendon sheath abnormalities.

5 The most serious forms of arthritis are rheumatoid arthritis, osteoarthritis, gout and systemic lupus erythematosus.

In addition to the rheumatic diseases, other diseases are characterized by inflammation. Gingivitis 10 and periodontitis follows a pathological picture resembling rheumatoid arthritis. Inflammatory bowel disease refers to idiopathic chronic inflammatory conditions of the intestine, ulcerative colitis and Crohn's disease. Spondylitis refers to chronic 15 inflammation of the joints of the spine. There is also a high incidence of osteoarthritis associated with obesity.

Thus, the inventive compounds have utility in the treatment of conditions involving inflammation, pain, 20 fever, fetal development, labor and platelet aggregation.

The inhibition of COX by the inventive compounds would also inhibit the formation of prostaglandins, e.g., PGD₂, PGE₂. Thus, the inventive compounds have utility in the treatment of conditions 25 associated with prostaglandin PGD₂, PGE₂.

NO-ASSOCIATED UTILITIES

Nitric oxide (NO) is known to inhibit platelet aggregation, monocyte adhesion and chemotaxis, and proliferation of vascular smooth muscle tissue which are 30 critically involved in the process of atherogenesis.

Evidence supports the view that NO is reduced in atherosclerotic tissues due to its reaction with oxygen free radicals. The loss of NO due to these reactions leads to increased platelet and inflammatory cell 35 adhesion to vessel walls to further impair NO mechanisms of relaxation. In this manner, the loss of NO promotes

atherogenic processes, leading to progressive disease states.

Hypertension is a leading cause of cardiovascular diseases, including stroke, heart attack, 5 heart failure, irregular heart beat and kidney failure. Hypertension is a condition where the pressure of blood within the blood vessels is higher than normal as it circulates through the body. When the systolic pressure exceeds 150 mm Hg or the diastolic pressure exceeds 90 mm 10 Hg for a sustained period of time, damage is done to the body. For example, excessive systolic pressure can rupture blood vessels anywhere. When it occurs within the brain, a stroke results. It can also cause thickening and narrowing of the blood vessels which can 15 lead to atherosclerosis. Elevated blood pressure can also force the heart muscle to enlarge as it works harder to overcome the elevated resting (diastolic) pressure when blood is expelled. This enlargement can eventually produce irregular heart beats or heart failure. 20 Hypertension is called the "silent killer" because it causes no symptoms and can only be detected when blood pressure is checked.

The regulation of blood pressure is a complex event where one mechanism involves the expression of 25 constitutive Ca^{+2} /calmodulin dependent form of nitric oxide synthase (NOS), abbreviated eNOS. NO produced by this enzyme produces muscle relaxation in the vessel (dilation), which lowers the blood pressure. When the normal level of NO produced by eNOS is not produced, 30 either because production is blocked by an inhibitor or in pathological states, such as atherosclerosis, the vascular muscles do not relax to the appropriate degree. The resulting vasoconstriction increases blood pressure and may be responsible for some forms of hypertension.

35 Vascular endothelial cells contain eNOS. NO synthesized by eNOS diffuses in diverse directions, and when it reaches the underlying vascular smooth muscle, NO

binds to the heme group of guanylyl cyclase, causing an increase in cGMP. Increased cGMP causes a decrease in intracellular free Ca⁺². Cyclic GMP may activate a protein kinase that phosphorylates Ca⁺² transporters, 5 causing Ca⁺² to be sequestered in intracellular structures in the muscle cells. Since muscle contraction requires Ca⁺², the force of the contraction is reduced as the Ca⁺² concentration declines. Muscle relaxation allows the vessel to dilate, which lowers the blood pressure.

10 Inhibition of eNOS therefore causes blood pressure to increase.

When the normal level of NO is not produced, either because production is blocked by administration of an NOS inhibitor or possibly, in pathological states, 15 such as atherosclerosis, the vascular muscles do not relax to the appropriate degree. The resulting vasoconstriction increases blood pressure and may be responsible for some forms of hypertension. There is considerable interest in finding therapeutic ways to 20 increase the activity of eNOS in hypertensive patients, but practical therapies have not been reported. Pharmacological agents capable of releasing NO, such as nitroglycerin or isosorbide dinitrate, remain mainstays of vasorelaxant therapy.

25 Although the inventive compounds inhibit the oxidation of LDL, the more comprehensive effects of these compounds is their multidimensional effects on atherosclerosis via NO. NO modulation by the inventive compounds brings about a collage of beneficial effects, 30 including the modulation of hypertension, lowering NO affected hypercholesterolemia, inhibiting platelet aggregation and monocyte adhesion, all of which are involved with the progression of atherosclerosis.

The role of NO in the immune system is 35 different from its function in blood vessels.

Macrophages contain a form of NOS that is inducible, rather than constitutive, referred to as iNOS.

Transcription of the iNOS gene is controlled both positively and negatively by a number of biological response modifiers called cytokines. The most important inducers are gamma-interferon, tumor necrosis factor, 5 interleukin-1, interleukin-2 and lipopolysaccharide (LPS), which is a component of the cell walls of gram negative bacteria. Stimulated macrophages produce enough NO to inhibit ribonuclease reductase, the enzyme that converts ribonucleotides to the deoxyribonucleotides 10 necessary for DNA synthesis. Inhibition of DNA synthesis may be an important way in which macrophages and other tissues possessing iNOS can inhibit the growth of rapidly dividing tumor cells or infectious bacteria.

With regard to the effects of NO and infectious 15 bacteria, microorganisms play a significant role in infectious processes which reflect body contact and injury, habits, profession, environment of the individual, as well as food borne diseases brought about by improper storage, handling and contamination.

20 The inventive compounds, combinations thereof and compositions containing the same are useful in the treatment of conditions associated with modulating NO concentrations. Example 9 described the antioxidant activity (as inhibitors of free radicals) of 25 the inventive compounds. Given that NO is a free radical and that the inventive compounds are strong antioxidants, it was suspected that the administration of the inventive compounds to experimental in vitro and in vivo models would have caused a reduction in NO levels. Any 30 reduction in NO would have resulted in a hypertensive, rather than a hypotensive effect. Contrary to expectations, the inventive compounds elicited increases in NO from in vitro experiments and produced a hypotensive effect from in vivo studies (Examples 31 and 35 32). These results were unanticipated and completely unexpected.

Example 27 describes an erythema (facial flush) shortly after drinking a solution containing the inventive compounds and glucose, thus implying a vasodilation effect.

5 Example 31 describes the hypotensive effects elicited by the inventive compounds in an *in vivo* animal model, demonstrating the efficacy of the inventive compounds in the treatment of hypertension. In this example, the inventive compounds, combinations thereof
10 and compositions comprising the same comprise oligomers wherein n is 2 to 18, and preferably, n is 2 to 10.

Example 32 describes the modulation of NO production by the inventive compounds in an *in vitro* model. In this example, the inventive compounds,
15 combinations thereof and compositions comprising the same comprise oligomers wherein n is 2 to 18, and preferably n is 2 to 10.

Further, Example 35 provides evidence for the formation of Cu⁺²-, Fe⁺²- and Fe⁺³-oligomer complexes
20 detected by MALDI/TOF/MS. These results indicate that the inventive compounds can complex with copper and/or iron ions to minimize their effects on LDL oxidation.

Moreover, the inventive compounds have useful anti-microbial activities for the treatment of infections
25 and for the prevention of food spoilage. Examples 22 and 30 describe the antimicrobial activity of the inventive compounds against several representative microbiota having clinical and food significance, as outlined below.

	MICROORGANISM	TYPE	CLINICAL/FOOD RELEVANCE
30	<i>Helicobacter pylori</i>	gram negative	gastritis, ulcers, gastric cancer

	<i>Bacillus</i> species	gram positive	food poisoning, wound infections, bovine mastitis, septicemia
	<i>Salmonella</i> species	gram negative	food poisoning, diarrhea
5	<i>Staphylococcus aureus</i>	gram positive	boils, carbuncles, wound infection, septicemia, breast abscesses
	<i>Escherichia coli</i>	gram negative	infant diarrhea, urinary tract infection
	<i>Pseudomonas</i> species	gram negative	urinary tract infections, wound infections, "swimmer's ear"
10	<i>Saccharomyces cervisea</i>	yeast	food spoilage
	<i>Acetobacter pasteurianus</i>	gram negative	food spoilage

Example 33 describes the effects of the
15 inventive compounds on macrophage NO production. In this
example, the results demonstrate that the inventive
compounds induce monocyte/macrophage NO production, both
independent and dependent of stimulation by
lipopolysaccharide (LPS) or cytokines. Macrophages
20 producing NO can inhibit the growth of infectious
bacteria.

Compounds of the invention eliciting
antimicrobial activity are oligomers, where n is 2 to 18,

and preferably, are oligomers where n is 2, 4, 5, 6, 8 and 10.

Examples of compounds eliciting the antimicrobial activity with respect to NO cited above 5 include dimers, tetramers, pentamers, hexamers, octamers and decamers, discussed above.

ANTI-CANCER UTILITIES

Cancers are classified into three groups: carcinomas, sarcomas and lymphomas. A carcinoma is a 10 malignancy that arises in the skin, linings of various organs, glands and tissues. A sarcoma is a malignancy that arises in the bone, muscle or connective tissue. The third group comprises leukemias and lymphomas because both develop within the blood cell forming organs. The 15 major types of cancer are prostate, breast, lung, colorectal, bladder, non-Hodgkin's lymphoma, uterine, melanoma of the skin, kidney, leukemia, ovarian and pancreatic.

The development of cancer results from 20 alterations to the DNA of cells which is brought about by many factors such as inheritable genetic factors, ionizing radiation, pollutants, radon, and free radical damage to the DNA. Cells carrying mutations produce a defect in the ordered process of cell division. These 25 cells fail to undergo apoptosis (programmed cell death) and continue to divide which either marks the beginnings of a malignant tumor or allows more mutations to occur over time to result in a malignancy.

There are three major features common to the 30 many different cancers. These are (1) the ability to proliferate indefinitely; (2) invasion of the tumor into the surrounding tissue; and (3) the process of metastasis.

Certain types of cancer metastasize in 35 characteristic ways. For example, cancers of the thyroid gland, lung, breast, kidney and prostate gland frequently metastasize to the bones. Lung cancer commonly spreads

to the brain and adrenal glands and colorectal cancer often metastasizes to the liver. Leukemia is considered to be a generalized disease at the onset, where it is found in the bone marrow throughout the body.

5 It has been surprisingly found that the inventive compounds are useful in the treatment of a variety of cancers discussed above. Examples 6, 7, 8 and 15 describe the inventive compounds which elicit anti-cancer activity against human HeLa (cervical), prostate, 10 breast, renal, T-cell leukemia and colon cancer cell lines. Example 12 (Fig. 20) illustrates the dose response effects on HeLa and SKBR-3 breast cancer cell lines treated with oligomeric (dimers - dodecamers) procyanidins, which were substantially purified by HPLC. 15 Cytotoxicity against these cancer cell lines were dependent upon pentamer through dodecamer procyanidins, with the lower oligomers showing no effect.

While not wishing to be bound by any theory, there appeared to be a minimum structural motif that 20 accounts for the effects described above. Example 37 also shows the same cytotoxic effects of the higher oligomers (pentamer - decamer) against a feline lymphoblastoid cancer cell line. Cytotoxicity was also observed with higher oligomers (Figs. 58 to 61) against 25 normal canine and feline cell lines.

In Example 8 (Figs. 9D-H), the inventive compounds were shown to elicit cytotoxicity against a putative COX-2 expressing human colon cancer cell line (HCT 116).

30 Example 9 describes the antioxidant activity by the inventive compounds. The compounds of the invention inhibit DNA strand breaks, DNA-protein cross-links and free radical oxidation of nucleotides to reduce and/or prevent the occurrence of mutations.

35 Example 10 describes the inventive compounds as topoisomerase II inhibitors, which is a target for chemotherapeutic agents, such as doxorubicin.

Example 21 describes the *in vivo* effects of a substantially pure pentamer which elicited anti-tumor activity against a human breast cancer cell line (MDA-MB-231/LCC6) in a nude mouse model (average weight of a 5 mouse is approximately 25g). Repeat *in vivo* experiments with the pentamer at higher dosages (5mg) have not entirely been successful, due to unexpected animal toxicity. It is currently believed that this toxicity may be related to the vasodilation effects of the 10 inventive compounds.

Example 33 describes the effects of the inventive compounds on macrophage NO production. Macrophages which produce NO can inhibit the growth of rapidly dividing tumor cells.

15 Still further, the invention includes the use of the inventive compounds to induce the inhibition of cellular proliferation by apoptosis.

For anti-cancer activity, the inventive compounds are oligomers, where n is 2 to 18, e.g., 3 to 20 18, such as 3 to 12, and preferably, n is 5 to 12, and most preferably n is 5.

Compounds which elicit the inhibitory activity with respect to cancer cited above include pentamers to dodecamers, discussed above.

25 FORMULATIONS AND METHODS

Therefore, collectively, the inventive compounds, combinations thereof and compositions comprising the same have exhibited a wide array of activities against several aspects of atherosclerosis, 30 cardiovascular disease, cancer, blood pressure modulation and/or hypertension, inflammatory disease, infectious agents and food spoilage.

Hence, the compounds of the invention, combinations thereof and compositions containing the same 35 are COX inhibitors which affect platelet aggregation by inhibiting thromboxane A₂ formation, thus reducing the risk for thrombosis. Further, the inhibition of COX

leads to decreased platelet and inflammatory cell adhesion to vessel walls to allow for improved NO mechanisms of relaxation. These results, coupled with the inhibition of COX at concentrations similar to a known NSAID, indomethacin, indicates antithrombotic efficacy.

Moreover, the compounds of the invention, combinations thereof and compositions containing the same are antioxidants which suppress the oxidation of LDL by reducing the levels of superoxide radical anion and lipoxygenase mediated lipid peroxy radicals. The inhibition of LDL oxidation at this stage slows macrophage activation and retards foam cell formation to interrupt further progression of atherosclerosis. The inhibition of LDL oxidation can also slow the progression of restenosis. Thus, compounds of the invention or combinations thereof or compositions containing compounds of the invention or combinations thereof can be used for prevention and/or treatment of atherosclerosis and/or restenosis. And thus, the inventive compounds can be administered before or after angioplasty or similar procedures to prevent or treat restenosis in patients susceptible thereto.

For treatment or prevention of restenosis and/or atherosclerosis, an inventive compound or compounds or a composition comprising an inventive compound or compounds, alone or with other treatment, may be administered as desired by the skilled medical practitioner, from this disclosure and knowledge in the art, e.g., at the first signs or symptoms of restenosis and/or atherosclerosis, immediately prior to, concomitant with or after angioplasty, or as soon thereafter as desired by the skilled medical practitioner, without any undue experimentation required; and the administration of the inventive compound or compounds or a composition thereof, alone or with other treatment, may be continued as a regimen, e.g., monthly, bi-monthly, biannually,

annually, or in some other regimen, by the skilled medical practitioner for such time as is necessary, without any undue experimentation required.

Further, the compounds of the invention, 5 combinations thereof and compositions comprising the same have been shown to produce a hypotensive effect *in vivo* and induce NO *in vitro*. These results have practical application in the treatment of hypertension and in clinical situations involving hypercholesterolemia, where 10 NO levels are markedly reduced.

Formulations of the inventive compounds, combinations thereof and compositions comprising the same can be prepared with standard techniques well known to those skilled in the pharmaceutical, food science, 15 medical and veterinary arts, in the form of a liquid, suspension, tablet, capsule, injectable solution or suppository, for immediate or slow-release of the active compounds.

The carrier may also be a polymeric delayed 20 release system. Synthetic polymers are particularly useful in the formulation of a composition having controlled release. An early example of this was the polymerization of methyl methacrylate into spheres having diameters less than one micron to form so-called nano 25 particles, reported by Kreuter, J., Microcapsules and Nanoparticles in Medicine and Pharmacology, M. Donbrow (Ed). CRC Press, p. 125-148.

A frequent choice of a carrier for pharmaceuticals and more recently for antigens is poly 30 (d,L-lactide-co-glycolide) (PLGA). This is a biodegradable polyester that has a long history of medical use in erodible sutures, bone plates and other temporary prostheses where it has not exhibited any toxicity. A wide variety of pharmaceuticals have been 35 formulated into PLGA microcapsules. A body of data has accumulated on the adaption of PLGA for controlled, for example, as reviewed by Eldridge, J.H., et al. Current

Topics in Microbiology and Immunology, 1989, 146:59-66.
The entrapment in PLGA microspheres of 1 to 10 microns in diameter can have an effect when administered orally.
The PLGA microencapsulation process uses a phase 5 separation of a water-in-oil emulsion. The inventive compound or compounds is or are prepared as an aqueous solution and the PLGA is dissolved in a suitable organic solvents such as methylene chloride and ethyl acetate.
These two immiscible solutions are co-emulsified by high-10 speed stirring. A non-solvent for the polymer is then added, causing precipitation of the polymer around the aqueous droplets to form embryonic microcapsules. The microcapsules are collected, and stabilized with one of an assortment of agents (polyvinyl alcohol (PVA), 15 gelatin, alginates, methyl cellulose) and the solvent removed by either drying in vacuo or solvent extraction.

Additionally, with regard to the preparation of slow-release formulations, reference is made to U.S. Patent Nos. 5,024,843, 5,091,190, 5,082,668, 4,612,008 20 and 4,327,725, hereby incorporated herein by reference.

Additionally, selective processing coupled with the identification of cocoa genotypes of interest could be used to prepare Standard-of-Identity (SOI) and non-SOI chocolate products as vehicles to deliver the active 25 compounds to a patient in need of treatment for the disease conditions described above, as well as a means for the delivery of conserved levels of the inventive compounds.

In this regard, reference is made to copending 30 U.S. Application Serial No. 08/709,406, filed September 6, 1996, hereby incorporated herein by reference. USSN 08/709,406 relates to a method of producing cocoa butter and/or cocoa solids having conserved levels of polyphenols from cocoa beans using a unique combination 35 of processing steps which does not require separate bean roasting or liquor milling equipment, allowing for the option of processing cocoa beans without exposure to

severe thermal treatment for extended periods of time and/or the use of solvent extraction of fat. The benefit of this process lies in the enhanced conservation of polyphenols in contrast to that found in traditional 5 cocoa processing, such that the ratio of the initial amount of polyphenol found in the unprocessed bean to that obtainable after processing is less than or equal to 2.

Compositions of the invention include one or 10 more of the above noted compounds in a formulation having a pharmaceutically acceptable carrier or excipient, the inventive compounds having anti-cancer, anti-tumor or antineoplastic activities, antioxidant activity, inhibit DNA topoisomerase II enzyme, inhibit oxidative damage to 15 DNA, induce monocyte/macrophage NO production, have antimicrobial, cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase, apoptosis, platelet aggregation and blood or *in vivo* glucose modulating activities, and have efficacy as non-steroidal antiinflammatory agents.

20 Another embodiment of the invention includes compositions comprising the inventive compounds or combinations thereof, as well as at least one additional antineoplastic, blood pressure reducing, antiinflammatory, antimicrobial, antioxidant and 25 hematopoiesis agents, in addition to a pharmaceutically acceptable carrier or excipient.

Such compositions can be administered to a subject or patient in need of such administration in dosages and by techniques well known to those skilled in 30 the medical, nutritional or veterinary arts taking into consideration the data herein, and such factors as the age, sex, weight, genetics and condition of the particular subject or patient, and the route of administration, relative concentration of particular 35 oligomers, and toxicity (e.g., LD₅₀).

The compositions can be co-administered or sequentially administered with other antineoplastic,

anti-tumor or anti-cancer agents, antioxidants, DNA topoisomerase II enzyme inhibiting agents, inhibitors of oxidatively damaged DNA or cyclo-oxygenase and/or lipoxygenase, apoptosis, platelet aggregation, blood or 5 in vivo glucose or NO or NO-synthase modulating agents, non-steroidal antiinflammatory agents and/or with agents which reduce or alleviate ill effects of antineoplastic, anti-tumor, anti-cancer agents, antioxidants, DNA topoisomerase II enzyme inhibiting agents, inhibitors of 10 oxidatively damaged DNA, cyclo-oxygenase and/or lipoxygenase, apoptosis, platelet aggregation, blood or in vivo glucose or NO or NO-synthase modulating and/or non-steroidal antiinflammatory agents; again, taking into consideration such factors as the age, sex, weight, 15 genetics and condition of the particular subject or patient, and, the route of administration.

Examples of compositions of the invention for human or veterinary use include edible compositions for oral administration, such solid or liquid formulations, 20 for instance, capsules, tablets, pills and the like, as well as chewable solid or beverage formulations, to which the present invention may be well-suited since it is from an edible source (e.g., cocoa or chocolate flavored solid or liquid compositions); liquid preparations for orifice, 25 e.g., oral, nasal, anal, vaginal etc., administration such as suspensions, syrups or elixirs (including cocoa or chocolate flavored compositions); and, preparations for parental, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable 30 administration) such as sterile suspensions or emulsions. However, the active ingredient in the compositions may complex with proteins such that when administered into the bloodstream, clotting may occur due to precipitation of blood proteins; and, the skilled artisan should take 35 this into account. In such compositions the active cocoa extract may be in admixture with a suitable carrier, diluent, or excipient such as sterile water,

physiological saline, glucose, DMSO, ethanol, or the like. The active cocoa extract of the invention can be provided in lyophilized form for reconstituting, for instance, in isotonic aqueous, saline, glucose or DMSO buffer. In certain saline solutions, some precipitation has been observed; and, this observation may be employed as a means to isolate inventive compounds, e.g., by a "salting out" procedure.

Example 38 describes the preparation of the inventive compounds in a tablet formulation for application in the pharmaceutical, supplement and food areas. Further, Example 39 describes the preparation of the inventive compounds in capsule formulations for similar applications. Still further, Example 40 describes the formulation of Standard of Identity (SOI) and non-SOI chocolates containing the compounds of the invention or cocoa solids obtained from methods described in copending U.S. Application Serial No. 08/709,406, hereby incorporated herein by reference.

20 KITS

Further, the invention also comprehends a kit wherein the active cocoa extract is provided. The kit can include a separate container containing a suitable carrier, diluent or excipient. The kit can also include an additional anti-cancer, anti-tumor or antineoplastic agent, antioxidant, DNA topoisomerase II enzyme inhibitor or an inhibitor of oxidative DNA damage or antimicrobial, or cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase non-steroidal antiinflammatory, apoptosis and platelet aggregation modulating or blood or *in vivo* glucose modulating agent and/or an agent which reduces or alleviates ill effects of antineoplastic, anti-tumor or anti-cancer agents, antioxidant, DNA topoisomerase II enzyme inhibitor or antimicrobial, or cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase, apoptosis, platelet aggregation and blood or *in vivo* glucose modulating and/or non-steroidal antiinflammatory agents.

for co- or sequential-administration. The additional agent(s) can be provided in separate container(s) or in admixture with the active cocoa extract. Additionally, the kit can include instructions for mixing or combining 5 ingredients and/or administration.

IDENTIFICATION OF GENES

A further embodiment of the invention comprehends the modulation of genes expressed as a result of intimate cellular contact by the inventive compounds 10 or a combination of compounds. As such, the present invention comprehends methods for the identification of genes induced or repressed by the inventive compounds or a combination of compounds which are associated with several diseases, including but not limited to 15 atherosclerosis, hypertension, cancer, cardiovascular disease, and inflammation. Specifically, genes which are differentially expressed in these disease states, relative to their expression in "normal" nondisease states are identified and described before and after 20 contact by the inventive compounds or a combination of compounds.

As mentioned in the previous discussion, these diseases and disease states are based in part on free radical interactions with a diversity of biomolecules. A 25 central theme in these diseases is that many of the free radical reactions involve reactive oxygen species, which in turn induce physiological conditions involved in disease progression. For instance, reactive oxygen species have been implicated in the regulation of 30 transcription factors such as nuclear factor (NF)- κ B. The target genes for NF- κ B comprise a list of genes linked to coordinated inflammatory response. These include genes encoding tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, IL-8, inducible NOS, Major 35 Histocompatibility Complex (MHC) class I antigens, and others. Also, genes that modulate the activity of transcription factors may in turn be induced by oxidative

stress. Oxidative stress is the imbalance between radical scavenging and radical generating systems. Several known examples (Winyard and Blake, 1997) of these conditions include gadd153 (a gene induced by growth 5 arrest and DNA damage), the product of which has been shown to bind NF-IL6 and form a heterodimer that cannot bind to DNA. NF-IL6 upregulates the expression of several genes, including those encoding interleukins 6 and 8. Another example of oxidative stress inducible 10 genes are gadd45 which regulates the effects of the transcription factor p53 in growth arrest. p53 codes for the p53 protein which can halt cell division and induce abnormal cells (e.g. cancer) to undergo apoptosis.

Given the full panoply of unexpected, 15 nonobvious and novel utilities for the inventive compounds or combination of compounds for utility in a diverse array of diseases based in part by free radical mechanisms, the invention further comprehends strategies to determine the temporal effects on gene(s) or gene 20 product(s) expression by the inventive compounds in animal *in vitro* and/or *in vivo* models of specific disease or disease states using gene expression assays. These assays include, but are not limited to Differential Display, sequencing of cDNA libraries, Serial Analysis of 25 Gene Expression (SAGE), expression monitoring by hybridization to high density oligonucleotide arrays and various reverse transcriptase-polymerization chain reaction (RT-PCR) based protocols or their combinations (Lockhart et al., 1996).

30 The comprehensive physiological effects of the inventive compounds or combination of compounds embodied in the invention, coupled to a genetic evaluation process permits the discovery of genes and gene products, whether known or novel, induced or repressed. For instance, the 35 invention comprehends the *in vitro* and *in vivo* induction and/or repression of cytokines (e.g. IL-1, IL-2, IL-6, IL-8, IL-12, and TNF- α) in lymphocytes using RT-PCR.

Similarly, the invention comprehends the application of Differential Display to ascertain the induction and/or repression of select genes; for the cardiovascular area (e.g. superoxide dismutase, heme oxidase, COX I and 2, 5 and other oxidant defense genes) under stimulated and/or oxidant stimulated conditions (e.g. TNF- α or H₂O₂) conditions. For the cancer area, the invention comprehends the application of Differential Display to ascertain the induction and/or repression of genes or 10 gene products such as CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase, etc., in control and oxidant stressed cells.

The following non-limiting Examples are given by way of illustration only and are not to be considered 15 a limitation of this invention, many apparent variations of which are possible without departing from the spirit or scope thereof.

EXAMPLES

20 **Example 1: Cocoa Source and Method of Preparation**

Several *Theobroma cacao* genotypes which represent the three recognized horticultural races of cocoa (Enriquez, 1967; Engels, 1981) were obtained from the three major cocoa producing origins of the world. A 25 list of those genotypes used in this study are shown in Table 1. Harvested cocoa pods were opened and the beans with pulp were removed for freeze drying. The pulp was manually removed from the freeze dried mass and the beans were subjected to analysis as follows. The unfermented, 30 freeze dried cocoa beans were first manually dehulled, and ground to a fine powdery mass with a TEKMAR Mill. The resultant mass was then defatted overnight by Soxhlet extraction using redistilled hexane as the solvent. Residual solvent was removed from the defatted mass by 35 vacuum at ambient temperature.

Table 1: Description of *Theobroma cacao* Source Material

GENOTYPE	ORIGIN	HORTICULTURAL RACE
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	UIT-1	Malaysia	Trinitario
	Unknown	West Africa	Forastero
5	ICS-100	Brazil	Trinitario (Nicaraguan Criollo ancestor)
	ICS-39	Brazil	Trinitario (Nicaraguan Criollo ancestor)
	UF-613	Brazil	Trinitario
	EEG-48	Brazil	Forastero
	UF-12	Brazil	Trinitario
	NA-33	Brazil	Forastero

10

Example 2: Procyanidin Extraction Procedures**A. Method 1**

Procyanidins were extracted from the defatted, unfermented, freeze dried cocoa beans of Example 1 using 15 a modification of the method described by Jalal and Collin (1977). Procyanidins were extracted from 50 gram batches of the defatted cocoa mass with 2X 400 mL 70% acetone/deionized water followed by 400mL 70% methanol/deionized water. The extracts were pooled and 20 the solvents removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was diluted to 1L with deionized water and extracted 2X with 400mL CHCl₃. The solvent phase was discarded. The aqueous phase was then extracted 4X with 25 500mL ethyl acetate. Any resultant emulsions were broken by centrifugation on a Sorvall RC 28S centrifuge operated at 2,000 xg for 30 min. at 10°C. To the combined ethyl

acetate extracts, 100-200mL deionized water was added. The solvent was removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was frozen in liquid N₂ followed by freeze drying on a LABCONCO Freeze Dry System. The yields of crude procyanidins that were obtained from the different cocoa genotypes are listed in Table 2.

Table 2: Crude Procyanidin Yields

	GENOTYPE	ORIGIN	YIELDS (g)
10	UIT-1	Malaysia	3.81
	Unknown	West Africa	2.55
	ICS-100	Brazil	3.42
	ICS-39	Brazil	3.45
	UF-613	Brazil	2.98
	EEG-48	Brazil	3.15
	UF-12	Brazil	1.21
	NA-33	Brazil	2.23

B. Method 2

20 Alternatively, procyanidins are extracted from defatted, unfermented, freeze dried cocoa beans of Example 1 with 70% aqueous acetone. Ten grams of defatted material was slurried with 100 mL solvent for 5-10 min. The slurry was centrifuged for 15 min. at 4°C at 25 3000 xg and the supernatant passed through glass wool. The filtrate was subjected to distillation under partial vacuum and the resultant aqueous phase frozen in liquid N₂, followed by freeze drying on a LABCONCO Freeze Dry System. The yields of crude procyanidins ranged from 15-30 20%.

Without wishing to be bound by any particular theory, it is believed that the differences in crude

yields reflected variations encountered with different genotypes, geographical origin, horticultural race, and method of preparation.

Example 3: Partial Purification of Cocoa Procyanidins

5 **A. Gel Permeation Chromatography**

Procyanidins obtained from Example 2 were partially purified by liquid chromatography on Sephadex LH-20 (28 x 2.5 cm). Separations were aided by a step gradient from deionized water into methanol. The initial 10 gradient composition started with 15% methanol in deionized water which was followed step wise every 30 min. with 25% methanol in deionized water, 35% methanol in deionized water, 70% methanol in deionized water, and finally 100% methanol. The effluent following the 15 elution of the xanthine alkaloids (caffeine and theobromine) was collected as a single fraction. The fraction yielded a xanthine alkaloid free subfraction which was submitted to further subfractionation to yield five subfractions designated MM2A through MM2E. The 20 solvent was removed from each subfraction by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was frozen in liquid N₂ and freeze dried overnight on a LABCONCO Freeze Dry System. A representative gel permeation chromatogram 25 showing the fractionation is shown in Figure 1.

Approximately, 100mg of material was subfractionated in this manner.

Chromatographic Conditions: Column; 28 x 2.5 cm Sephadex 30 LH-20, Mobile Phase: Methanol/Water Step Gradient, 15:85, 25:75, 35:65, 70:30, 100:0 Stepped at 1/2 Hour Intervals, Flow Rate; 1.5mL/min, Detector; UV at $\lambda_1 = 254$ nm and $\lambda_2 = 365$ nm, Chart Speed: 0.5mm/min, Column Load; 120mg.

35 **B. Semi-preparative High Performance Liquid**

Chromatography (HPLC)

Method 1. Reverse Phase Separation

Procyanidins obtained from Example 2 and/or 3A were partially purified by semi-preparative HPLC. A Hewlett Packard 1050 HPLC System equipped with a variable wavelength detector, Rheodyne 7010 injection valve with 1mL injection loop was assembled with a Pharmacia FRAC-100 Fraction Collector. Separations were effected on a Phenomenex Ultracarb™ 10 μ ODS column (250 x 22.5mm) connected with a Phenomenex 10 μ ODS Ultracarb™ 10 (60 x 10 mm) guard column. The mobile phase composition was A = water; B = methanol used under the following linear gradient conditions: [Time, %A]; (0,85), (60,50), (90,0), and (110,0) at a flow rate of 5mL/min. Compounds were detected by UV at 254nm

A representative Semi-preparative HPLC trace is shown in Figure 15N for the separation of procyanidins present in fraction D + E. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further purification and subsequent evaluation. Injection loads ranged from 25-100mg of material.

Method 2. Normal Phase Separation

Procyanidin extracts obtained from Examples 2 and/or 3A were partially purified by semi-preparative HPLC. A Hewlett Packard 1050 HPLC system, Millipore-Waters Model 480 LC detector set at 254nm was assembled with a Pharmacia Frac-100 Fraction Collector set in peak mode. Separations were effected on a Supelco 5μm Supelcosil LC-Si column (250 x 10mm) connected with a Supelco 5μm Supelguard LC-Si guard column (20 x 4.6mm). Procyanidins were eluted by a linear gradient under the following conditions: (Time, %A, %B); (0,82,14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by a 10 min. re-equilibration. Mobile phase composition was A = dichloromethane; B = methanol; and C = acetic acid: water (1:1). A flow rate of 3mL/min was used. Components were detected by UV at 254nm, and

recorded on a Kipp & Zonan BD41 recorder. Injection volumes ranged from 100-250 μ L of 10mg of procyanidin extracts dissolved in 0.25mL 70% aqueous acetone. A representative semi-preparative HPLC trace is shown in 5 Figure 15 O. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further purification and subsequent evaluation.

HPLC Conditions: 250 x 10mm Supelco Supelcosil LC-Si
10 (5 μ m) Semipreparative Column

20 x 4.6mm Supelco Supelcosil LC-Si
(5 μ m) Guard Column

Detector: Waters LC

Spectrophotometer Model

15 480 @ 254nm

Flow rate: 3mL/min,

Column Temperature: ambient,

Injection: 250 μ L of 70% aqueous
acetone extract.

20	Gradient: Time (min)	CH ₂ Cl ₂	Methanol	Acetic Acid:H ₂ O (1:1)
	0	82	14	4
	30	67.6	28.4	4
	60	46	50	4
25	65	10	86	4
	70	10	86	4

The fractions obtained were as follows:

<u>FRACTION</u>	<u>TYPE</u>
1	dimers
2	trimers
5	tetramers
4	pentamers
5	hexamers
6	heptamers
7	octamers
10	nonamers
8	decamers
9	undecamers
10	dodecamers
12	higher oligomers

15

Example 4: Analytical HPLC Analysis of Procyanidin Extracts

Method 1. Reverse Phase Separation

Procyanidin extracts obtained from Example 3
 20 were filtered through a 0.45μ filter and analyzed by a
 Hewlett Packard 1090 ternary HPLC system equipped with a
 Diode Array detector and a HP model 1046A Programmable
 Fluorescence Detector. Separations were effected at 45°C
 on a Hewlett-Packard 5μ Hypersil ODS column ($200 \times$
 25 2.1mm). The flavanols and procyanidins were eluted with
 a linear gradient of 60% B into A followed by a column
 wash with B at a flow rate of 0.3mL/min . The mobile
 phase composition was B = 0.5% acetic acid in methanol
 and A = 0.5% acetic acid in nanopure water. Acetic acid

levels in A and B mobile phases can be increased to 2%. Components were detected by fluorescence, where $\lambda_{ex} = 276\text{nm}$ and $\lambda_{em} = 316\text{nm}$ and by UV at 280nm. Concentrations of (+)-catechin and (-)-epicatechin were determined relative to reference standard solutions. Procyanidin levels were estimated by using the response factor for (-)-epicatechin. A representative HPLC chromatogram showing the separation of the various components is shown in Figure 2A for one cocoa genotype. Similar HPLC profiles were obtained from the other cocoa genotypes.

HPLC Conditions: Column: 200 x 2.1mm Hewlett Packard Hypersil ODS (5 μ)

Guard column: 20 x 2.1mm Hewlett Packard Hypersil ODS (5 μ)

15 Detectors: Diode Array @ 280nm

Fluorescence $\lambda_{ex} = 276\text{nm}$;
 $\lambda_{em} = 316\text{nm}$.

Flow rate: 0.3mL/min.

Column Temperature: 45°C

20	Gradient: Time (min)	0.5% Acetic Acid in nanopure water	0.5% Acetic acid in methanol
	0	100	0
	50	40	60
	60	0	100

Method 2. Normal Phase Separation

Procyanidin extracts obtained from Examples 2 and/or 3 were filtered through a 0.45μ filter and analyzed by a Hewlett Packard 1090 Series II HPLC system 5 equipped with a HP model 1046A Programmable Fluorescence detector and Diode Array detector. Separations were effected at 37°C on a 5μ Phenomenex Lichrosphere[®] Silica 100 column ($250 \times 3.2\text{mm}$) connected to a Supelco Supelguard LC-Si 5μ guard column ($20 \times 4.6\text{mm}$).
10 Procyanidins were eluted by linear gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by an 8 min. re-equilibration. Mobile phase composition was A=dichloromethane, B=methanol, and
15 C=acetic acid: water at a volume ratio of 1:1. A flow rate of 0.5 mL/min. was used. Components were detected by fluorescence, where $\lambda_{\text{ex}} = 276\text{nm}$ and $\lambda_{\text{em}} = 316\text{nm}$ or by UV at 280 nm. A representative HPLC chromatogram showing the separation of the various procyanidins is shown in
20 Figure 2B for one genotype. Similar HPLC profiles were obtained from other cocoa genotypes.

HPLC Conditions:

25 250 x 3.2mm Phenomenex Lichrosphere[®] Silica 100
 column (5μ) 20 x 4.6mm
 Supelco Supelguard
 LC-Si (5μ) guard column
Detectors: Photodiode Array @ 280nm
 Fluorescence $\lambda_{\text{ex}} = 276\text{nm}$;
 $\lambda_{\text{em}} = 316\text{nm}$.
30 Flow rate: 0.5 mL/min.
 Column Temperature: 37°C

Gradient: Time (min.)	CH ₂ -Cl ₂	Methanol	Acetic Acid/Water (1:1)
5	82	14	4
	30	67.6	28.4
	60	46	50
	65	10	86
	70	10	86

10 Example 5: Identification of Procyanidins

Procyanidins were purified by liquid chromatography on Sephadex LH-20 (28 x 2.5cm) columns followed by semi-preparative HPLC using a 10 μ Bondapak C18 (100 x 8mm) column or by semi-preparative HPLC using 15 a 5 μ Supelcosil LC-Si (250 x 10mm) column.

Partially purified isolates were analyzed by Fast Atom Bombardment - Mass Spectrometry (FAB-MS) on a VG ZAB-T high resolution MS system using a Liquid Secondary Ion Mass Spectrometry (LSIMS) technique in 20 positive and negative ion modes. A cesium ion gun was used as the ionizing source at 30kV and a "Magic Bullet Matrix" (1:1 dithiothreitol/dithioerythritol) was used as the proton donor.

Analytical investigations of these fractions by 25 LSIMS revealed the presence of a number of flavan-3-ol oligomers as shown in Table

Table 3: LSIMS (Positive Ion) Data from Cocoa Procyanidin Fractions

Oligomer	$(M + 1)^+$ <i>m/z</i>	$(M + Na)^+$ <i>m/z</i>	Mol. Wt.
Monomers (catechins)	291	313	290
Dimer(s)	577/579	599/601	576/578
Trimer(s)	865/867	887/889	864/866
Tetramer(s)	1155	1177	1154
Pentamer(s)	1443	1465	1442
Hexamer(s)	1731	1753	1730
Heptamer(s)	---	2041	2018
Octamer(s)	---	2329	2306
Nonamer(s)	---	2617	2594
Decamer(s)	---	2905	2882
Undecamer(s)	---	---	3170
Dodecamer(s)	---	---	3458

The major mass fragment ions were consistent with work previously reported for both positive and negative ion FAB-MS analysis of procyanidins (Self et al., 1986 and Porter et al., 1991). The ion corresponding to m/z 577 ($M+H$) $^+$ and its sodium adduct at m/z 599 ($M+Na$) $^+$ suggested the presence of doubly linked procyanidin dimers in the isolates. It was interesting to note that the higher oligomers were more likely to form sodium adducts ($M+Na$) $^+$ than their protonated molecular ions ($M+H$) $^+$. The procyanidin isomers B-2, B-5 and C-1 were tentatively identified based on the work reported by Revilla et al. (1991), Self et al. (1986) and Porter et al. (1991). Procyanidins up to both the octamer and decamer were verified by FAB-MS in the

partially purified fractions. Additionally, evidence for procyanidins up to the dodecamer were observed from normal phase HPLC analysis (see Figure 2B). Table 4 lists the relative concentrations of the procyanidins found in xanthine alkaloid free isolates based on reverse phase HPLC analysis. Table 5 lists the relative concentrations of the procyanidins based on normal phase HPLC analysis.

Table 4: Relative Concentrations of Procyanidins in the Xanthine Alkaloid Free Isolates

Component	Amount
(+)-catechin	1.6%
(-)-epicatechin	38.2%
B-2 Dimer	11.0%
B-5 Dimer	5.3%
C-1 Trimer	9.3%
Doubly linked dimers	3.0%
Tetramer(s)	4.5%
Pentamer-Octamer	24.5%
Unknowns and higher oligomers	2.6%

Table 5: Relative Concentrations of Procyanidins in Aqueous Acetone Extracts

	Component	Amount
5	(+)-catechin and (-) -epicatechin	41.9%
	B-2 and B-5 Dimers	13.9%
	Trimers	11.3%
	Tetramers	9.9%
10	Pentamers	7.8%
	Hexamers	5.1%
	Heptamers	4.2%
	Octamers	2.8%
	Nonamers	1.6%
15	Decamers	0.7%
	Undecamers	0.2%
	Dodecamers	<0.1%

Figure 3 shows several procyanidin structures
 20 and Figures 4A-4E show the representative HPLC chromatograms of the five fractions employed in the following screening for anti-cancer or antineoplastic activity. The HPLC conditions for Figs. 4A-4E were as follows:

25 HPLC Conditions: Hewlett Packard 1090 ternary HPLC System equipped with HP Model 1046A Programmable Fluorescence Detector.
 Column: Hewlett Packard 5 μ Hypersil ODS (200 x 2.1mm) Linear Gradient of 60% B into A at a flow rate of 30 0.3mL/min. B = 0.5% acetic acid in methanol; A = 0.5% acetic acid in deionized water. λ_{ex} = 280nm; λ_{em} = 316nm.

Figure 15 O shows a representative semi-prep HPLC chromatogram of an additional 12 fractions employed in the screening for anticancer or antineoplastic activity (HPLC conditions stated above).

5 **Example 6: Anti-Cancer, Anti-Tumor or Antineoplastic Activity of Cocoa Extracts (Procyanidins)**

The MTT (3-[4,5-dimethyl thiazol-2yl]-2,5-diphenyltetrazolium bromide) - microtiter plate 10 tetrazolium cytotoxicity assay originally developed by Mosmann (1983) was used to screen test samples from Example 5. Test samples, standards (cisplatin and chlorambucil) and MTT reagent were dissolved in 100% DMSO (dimethyl sulfoxide) at a 10mg/mL concentration. Serial 15 dilutions were prepared from the stock solutions. In the case of the test samples, dilutions ranging from 0.01 through 100 μ g/mL were prepared in 0.5% DMSO.

All human tumor cell lines were obtained from the American Type Culture Collection. Cells were grown 20 as mono layers in alpha-MEM containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin and 240 units/mL nystatin. The cells were maintained in a humidified, 5% CO₂ atmosphere at 37°C.

After trypsinization, the cells are counted and 25 adjusted to a concentration of 50 x 10⁵ cells/mL (varied according to cancer cell line). 200 μ L of the cell suspension was plated into wells of 4 rows of a 96-well microtiter plate. After the cells were allowed to attach for four hours, 2 μ L of DMSO containing test sample 30 solutions were added to quadruplicate wells. Initial dose-response finding experiments, using order of magnitude test sample dilutions were used to determine the range of doses to be examined. Well absorbencies at 540nm were then measured on a BIO RAD MP450 plate reader. 35 The mean absorbance of quadruplicate test sample treated wells was compared to the control, and the results expressed as the percentage of control absorbance plus/minus the standard deviation. The reduction of MTT

to a purple formazan product correlates in a linear manner with the number of living cells in the well. Thus, by measuring the absorbance of the reduction product, a quantitation of the percent of cell survival 5 at a given dose of test sample can be obtained. Control wells contained a final concentration of 1% DMSO.

Two of the samples were first tested by this protocol. Sample MM1 represented a very crude isolate of cocoa procyanidins and contained appreciable quantities 10 of caffeine and theobromine. Sample MM2 represented a cocoa procyanidin isolate partially purified by gel permeation chromatography. Caffeine and theobromine were absent in MM2. Both samples were screened for activity against the following cancer cell lines using the 15 procedures previously described:

HCT 116 colon cancer
ACHN renal adenocarcinoma
SK-5 melanoma
A498 renal adenocarcinoma
20 MCF-7 breast cancer
PC-3 prostate cancer
CAPAN-2 pancreatic cancer

Little or no activity was observed with MM1 on 25 any of the cancer cell lines investigated. MM2 was found to have activity against HCT-116, PC-3 and ACHN cancer cell lines. However, both MM1 and MM2 were found to interfere with MTT such that it obscured the decrease in absorbance that would have reflected a decrease in viable 30 cell number. This interference also contributed to large error bars, because the chemical reaction appeared to go more quickly in the wells along the perimeter of the plate. A typical example of these effects is shown in Figure 5. At the high concentrations of test material, 35 one would have expected to observe a large decrease in survivors rather than the high survivor levels shown. Nevertheless, microscopic examinations revealed that

cytotoxic effects occurred, despite the MTT interference effects. For instance, an IC₅₀ value of 0.5 μ g/mL for the effect of MM2 on the ACHN cell line was obtained in this manner.

5 These preliminary results, in the inventors' view, required amendment of the assay procedures to preclude the interference with MTT. This was accomplished as follows. After incubation of the plates at 37°C in a humidified, 5% CO₂ atmosphere for 18 hours, 10 the medium was carefully aspirated and replaced with fresh alpha-MEM media. This media was again aspirated from the wells on the third day of the assay and replaced with 100 μ L of freshly prepared McCoy's medium. 11 μ L of a 5mg/mL stock solution of MTT in PBS (Phosphate Buffered 15 Saline) were then added to the wells of each plate. After incubation for 4 hours in a humidified, 5% CO₂ atmosphere at 37°C, 100 μ L of 0.04 N HCl in isopropanol was added to all wells of the plate, followed by thorough mixing to solubilize the formazan produced by any viable 20 cells. Additionally, it was decided to subfractionate the procyanidins to determine the specific components responsible for activity.

The subfractionation procedures previously described were used to prepare samples for further 25 screening. Five fractions representing the areas shown in Figure 1 and component(s) distribution shown in Figures 4A - 4E were prepared. The samples were coded MM2A through MM2E to reflect these analytical characterizations and to designate the absence of 30 caffeine and theobromine.

Each fraction was individually screened against the HCT-116, PC-3 and ACHN cancer cell lines. The results indicated that the activity did not concentrate to any one specific fraction. This type of result was 35 not considered unusual, since the components in "active" natural product isolates can behave synergistically. In the case of the cocoa procyanidin isolate (MM2), over

twenty detectable components comprised the isolate. It was considered possible that the activity was related to a combination of components present in the different fractions, rather than the activity being related to an individual component(s).

On the basis of these results, it was decided to combine the fractions and repeat the assays against the same cancer cell lines. Several fraction combinations produced cytotoxic effects against the PC-3 cancer cell lines. Specifically, IC₅₀ values of 40µg/mL each for MM2A and MM2E combination, and of 20µg/mL each for MM2C and MM2E combination, were obtained. Activity was also reported against the HCT-116 and ACHN cell lines, but as before, interference with the MTT indicator precluded precise observations. Replicate experiments were repeatedly performed on the HCT-116 and ACHN lines to improve the data. However, these results were inconclusive due to bacterial contamination and exhaustion of the test sample material. Figures 6A-6D show the dose-response relationship between combinations of the cocoa extracts and PC-3 cancer cells.

Nonetheless, from this data, it is clear that cocoa extracts, especially cocoa polyphenols or procyanidins, have significant anti-tumor; anti-cancer or antineoplastic activity, especially with respect to human PC-3 (prostate), HCT-116 (colon) and ACHN (renal) cancer cell lines. In addition, those results suggest that specific procyanidin fractions may be responsible for the activity against the PC-3 cell line.

30 Example 7: Anti-Cancer, Anti-Tumor or Antineoplastic Activity of Cocoa Extracts (Procyanidins)

To confirm the above findings and further study fraction combinations, another comprehensive screening was performed.

All prepared materials and procedures were identical to those reported above, except that the

standard 4-replicates per test dose was increased to 8 or 12-replicates per test dose. For this study, individual and combinations of five cocoa procyanidin fractions were screened against the following cancer cell lines.

5	PC-3 Prostate
	KB Nasopharyngeal/HeLa
	HCT-116 Colon
	ACHN Renal
	MCF-7 Breast
10	SK-5 Melanoma
	A-549 Lung
	CCRF-CEM T-cell leukemia

Individual screenings consisted of assaying 15 different dose levels (0.01-100 μ g/mL) of fractions A, B, C, D, and E (See Figs. 4A-4E and discussion thereof, supra) against each cell line. Combination screenings consisted of combining equal dose levels of fractions A+B, A+C, A+D, A+E, B+C, B+D, B+E, C+D, C+E, and D+E 20 against each cell line. The results from these assays are individually discussed, followed by an overall summary.

A. PC-3 Prostate Cell Line

Figures 7A - 7H show the typical dose response 25 relationship between cocoa procyanidin fractions and the PC-3 cell line. Figures 7D and 7E demonstrate that fractions D and E were active at an IC₅₀ value of 75 μ g/mL. The IC₅₀ values that were obtained from dose-response curves of the other procyanidin fraction combinations 30 ranged between 60 - 80 μ g/mL when fractions D or E were present. The individual IC₅₀ values are listed in Table 6.

B. KB Nasopharyngeal/HeLa Cell Line

Figures 8A - 8H show the typical dose response 35 relationship between cocoa procyanidin fractions and the KB Nasopharyngeal/HeLa cell line. Figures 8D and 8E demonstrate that fractions D and E were active at an IC₅₀

value of 75 μ g/mL. Figures 8F - 8H depict representative results obtained from the fraction combination study. In this case, procyanidin fraction combination A+B had no effect, whereas fraction combinations B+E and D+E were 5 active at an IC₅₀ value of 60 μ g/mL. The IC₅₀ values that were obtained from other dose response curves from other fraction combinations ranged from 60 - 80 μ g/mL when fractions D or E were present. The individual IC₅₀ values are listed in Table 6. These results were essentially 10 the same as those obtained against the PC-3 cell line.

C. HCT-116 Colon Cell Line

Figure 9A - 9H show the typical dose response relationships between cocoa procyanidin fractions and the HCT-116 colon cell line. Figures 9D and 9E demonstrate 15 that fraction E was active at an IC₅₀ value of approximately 400 μ g/mL. This value was obtained by extrapolation of the existing curve. Note that the slope of the dose response curve for fraction D also indicated activity. However, no IC₅₀ value was determined from this 20 plot, since the slope of the curve was too shallow to obtain a reliable value. Figures 9F - 9H depict representative results obtained from the fraction combination study. In this case, procyanidin fraction combination B+D did not show appreciable activity, 25 whereas fraction combinations A+E and D+E were active at IC₅₀ values of 500 μ g/mL and 85 μ g/mL, respectively. The IC₅₀ values that were obtained from dose response curves of other fraction combinations averaged about 250 μ g/mL when fraction E was present. The extrapolated IC₅₀ values 30 are listed in Table 6.

D. ACHN Renal Cell Line

Figure 10A - 10H show the typical dose response relationships between cocoa procyanidin fractions and the ACHN renal cell line. Figures 10A - 10E indicated that 35 no individual fraction was active against this cell line. Figures 10F - 10H depict representative results obtained from the fraction combination study. In this case,

procyanidin fraction combination B+C was inactive, whereas the fraction combination A+E resulted in an extrapolated IC₅₀ value of approximately 500µg/mL. Dose response curves similar to the C+D combination were 5 considered inactive, since their slopes were too shallow. Extrapolated IC₅₀ values for other fraction combinations are listed in Table 6.

E. A-549 Lung Cell Line

Figures 11A - 11H show the typical dose 10 response relationships between cocoa procyanidin fractions and the A-549 lung cell line. No activity could be detected from any individual fraction or combination of fractions at the doses used in the assay. However, procyanidin fractions may nonetheless have 15 utility with respect to this cell line.

F. SK-5 Melanoma Cell Line

Figure 12A - 12H show the typical dose response relationships between cocoa procyanidin fractions and the SK-5 melanoma cell line. No activity could be detected 20 from any individual fraction or combination of fractions at the doses used in the assay. However, procyanidin fractions may nonetheless have utility with respect to this cell line.

G. MCF-7 Breast Cell Line

25 Figures 13A - 13H show the typical dose response relationships between cocoa procyanidin fractions and the MCF-7 breast cell line. No activity could be detected from any individual fraction or combination of fractions at the doses used in the assay. 30 However, procyanidin fractions may nonetheless have utility with respect to this cell line.

H. CCRF-CEM T-Cell Leukemia Line

A typical dose response curves were originally obtained against the CCRF-CEM T-cell leukemia line. However, microscopic counts of cell number versus time at 5 different fraction concentrations indicated that 500 µg of fractions A, B and D effected an 80% growth reduction over a four day period. A representative dose response relationship is shown in Figure 14.

I. Summary

10 The IC₅₀ values obtained from these assays are collectively listed in Table 6 for all the cell lines except for CCRF-CEM T-cell leukemia. The T-cell leukemia data was intentionally omitted from the Table, since a different assay procedure was used. A general summary of 15 these results indicated that the most activity was associated with fractions D and E. These fractions were most active against the PC-3 (prostate) and KB (nasopharyngeal/HeLa) cell lines. These fractions also evidenced activity against the HCT-116 (colon) and ACHN 20 (renal) cell lines, albeit but only at much higher doses. No activity was detected against the MCF-7 (breast), SK-5 (melanoma) and A-549 (lung) cell lines. However, procyanidin fractions may nonetheless have utility with respect to these cell lines. Activity was also shown 25 against the CCRF-CEM (T-cell leukemia) cell line. It should also be noted that fractions D and E are the most complex compositionally. Nonetheless, from this data it is clear that cocoa extracts, especially cocoa procyanidins, have significant anti-tumor, anti-cancer or 30 antineoplastic activity.

**Table 6: IC₅₀ Values for Cocoa Procyanidin Fractions
Against Various Cell Lines**

(IC₅₀ values in µg/mL)

5

FRACTION	PC-3	KB	HCT-116	ACHN	MCF-7	SK-5	A-549
A							
B							
C							
D	90	80					
E	75	75	400				
A+B							
A+C	125	100					
A+D	75	75					
A+E	80	75	500	500			
B+C							
B+D	75	80					
B+E	60	65	200				
C+D	80	75		1000			
C+E	80	70	250				
D+E	80	60	85				

Values above 100µg/mL were extrapolated from
dose response curves

**Example 8. Anti-Cancer, Anti-Tumor or Antineoplastic
Activity of Cocoa Extracts (Procyanidins)**

5 Several additional *in vitro* assay procedures
were used to complement and extend the results presented
in Examples 6 and 7.

Method A. Crystal Violet Staining Assay

10 All human tumor cell lines were obtained from
the American Type Culture Collection. Cells were grown
as monolayers in IMEM containing 10% fetal bovine serum
without antibiotics. The cells were maintained in a
humidified, 5% CO₂ atmosphere at 37°C.

15 After trypsinization, the cells were counted
and adjusted to a concentration of 1,000-2,000 cells per
100 mL. Cell proliferation was determined by plating the
cells (1,000-2,000 cells/well) in a 96 well microtiter
plate. After addition of 100µL cells per well, the cells
were allowed to attach for 24 hours. At the end of the
20 24 hour period, various cocoa fractions were added at
different concentrations to obtain dose response results.
The cocoa fractions were dissolved in media at a 2 fold
concentration and 100µL of each solution was added in
triplicate wells. On consecutive days, the plates were
25 stained with 50µL crystal violet (2.5g crystal violet
dissolved in 125mL methanol, 375mL water), for 15 min.
The stain was removed and the plate was gently immersed
into cold water to remove excess stain. The washings
were repeated two more times, and the plates allowed to
30 dry. The remaining stain was solubilized by adding 100µL
of 0.1M sodium citrate/50% ethanol to each well. After
solubilization, the number of cells were quantitated on
an ELISA plate reader at 540nm (reference filter at
410nm). The results from the ELISA reader were graphed
35 with absorbance on the y-axis and days growth on the x-
axis.

Method B. Soft Agar Cloning Assay

Cells were cloned in soft agar according to the method described by Nawata et al. (1981). Single cell suspensions were made in media containing 0.8% agar with various concentrations of cocoa fractions. The 5 suspensions were aliquoted into 35mm dishes coated with media containing 1.0% agar. After 10 days incubation, the number of colonies greater than 60 μ m in diameter were determined on an Ominicron 3600 Image Analysis System. The results were plotted with number of colonies on the 10 y-axis and the concentrations of a cocoa fraction on the x-axis.

Method C. XTT-Microculture Tetrazolium Assay

The XTT assay procedure described by Scudiero et al. (1988) was used to screen various cocoa fractions. 15 The XTT assay was essentially the same as that described using the MTT procedure (Example 6) except for the following modifications. XTT ((2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino)carbonyl)-2H-tetrazolium hydroxide) was prepared at 1mg/mL medium 20 without serum, prewarmed to 37°C. PMS was prepared at 5mM PBS. XTT and PMS were mixed together; 10 μ L of PMS per mL XTT and 50 μ L PMS-XTT were added to each well. After an incubation at 37°C for 4 hr, the plates were 25 mixed 30 min. on a mechanical shaker and the absorbance measured at 450-600nm. The results were plotted with the absorbance on the y-axis and days growth or concentration on the x-axis.

For methods A and C, the results were also plotted as the percent control as the y-axis and days 30 growth or concentration on the x-axis.

A comparison of the XTT and Crystal Violet Assay procedures was made with cocoa fraction D & E (Example 3B) against the breast cancer cell line MCF-7 p168 to determine which assay was most sensitive. As 35 shown in Figure 15A, both assays showed the same dose-response effects for concentrations >75 μ g/mL. At concentrations below this value, the crystal violet assay

showed higher standard deviations than the XTT assay results. However, since the crystal violet assay was easier to use, all subsequent assays, unless otherwise specified, were performed by this procedure.

5 Crystal violet assay results are presented (Figures 15B-15E) to demonstrate the effect of a crude polyphenol extract (Example 2) on the breast cancer cell line MDA MB231, prostate cancer cell line PC-3, breast cancer cell line MCF-7 p163, and cervical cancer cell 10 line Hela, respectively. In all cases a dose of 250 μ g/mL completely inhibited all cancer cell growth over a period of 5-7 days. The Hela cell line appeared to be more sensitive to the extract, since a 100 μ g/mL dose also inhibited growth. Cocoa fractions from Example 3B were 15 also assayed against Hela and another breast cancer cell line SKBR-3. The results (Figures 15F and 15G) showed that fraction D & E has the highest activity. As shown in Figures 15H and 15I, IC₅₀ values of about 40 μ g/mL D & E were obtained from both cancer cell lines.

20 The cocoa fraction D & E was also tested in the soft agar cloning assay which determines the ability of a test compound(s) to inhibit anchorage independent growth. As shown in Figure 15J, a concentration of 100 μ g/mL completely inhibited colony formation of Hela cells.

25 Crude polyphenol extracts obtained from eight different cocoa genotypes representing the three horticultural races of cocoa were also assayed against the Hela cell line. As shown in Figure 15K all cocoa varieties showed similar dose-response effects. The UIT-30 1 variety exhibited the most activity against the Hela cell line. These results demonstrated that all cocoa genotypes possess a polyphenol fraction that elicits activity against at least one human cancer cell line that is independent of geographical origin, horticultural 35 race, and genotype.

Another series of assays were performed on crude polyphenol extracts prepared on a daily basis from

a one ton scale traditional 5-day fermentation of Brazilian cocoa beans, followed by a 4-day sun drying stage. The results shown in Figure 15L showed no obvious effect of these early processing stages, suggesting little change in the composition of the polyphenols. However, it is known (Lehrian and Patterson, 1983) that polyphenol oxidase (PPO) will oxidize polyphenols during the fermentation stage. To determine what effect enzymatically oxidized polyphenols would have on activity, another experiment was performed. Crude PPO was prepared by extracting finely ground, unfermented, freeze dried, defatted Brazilian cocoa beans with acetone at a ratio of 1gm powder to 10mL acetone. The slurry was centrifuged at 3,000 rpm for 15 min. This was repeated three times, discarding the supernatant each time with the fourth extraction being poured through a Buchner filtering funnel. The acetone powder was allowed to air dry, followed by assay according to the procedures described by McLord and Kilara, (1983). To a solution of crude polyphenols (100mg/10mL Citrate-Phosphate buffer, 0.02M, pH 5.5) 100mg of acetone powder (4,000 units activity/mg protein) was added and allowed to stir for 30 min. with a stream of air bubbled through the slurry. The sample was centrifuged at 5,000xg for 15 min. and the supernatant extracted 3X with 20mL ethyl acetate. The ethyl acetate extracts were combined, taken to dryness by distillation under partial vacuum and 5mL water added, followed by lyophilization. The material was then assayed against Hela cells and the dose-response compared to crude polyphenol extracts that were not enzymatically treated. The results (Figure 15M) showed a significant shift in the dose-response curve for the enzymatically oxidized extract, showing that the oxidized products were more inhibitory than their native forms.

**Example 9: Antioxidant Activity of Cocoa Extracts
Containing Procyanidins**

Evidence in the literature suggests a relationship between the consumption of naturally occurring antioxidants (Vitamins C, E and Beta-carotene) and a lowered incidence of disease, including cancer (Designing Foods, 1993; Caragay, 1992). It is generally thought that these antioxidants affect certain oxidative and free radical processes involved with some types of tumor promotion. Additionally, some plant polyphenolic compounds that have been shown to be anticarcinogenic, also possess substantial antioxidant activity (Ho et al., 1992; Huang et al., 1992).

To determine whether cocoa extracts containing procyanidins possessed antioxidant properties, a standard Rancimat method was employed. The procedures described in Examples 1, 2 and 3 were used to prepare cocoa extracts which were manipulated further to produce two fractions from gel permeation chromatography. These two fractions are actually combined fractions A through C, and D and E (See Figure 1) whose antioxidant properties were compared against the synthetic antioxidants BHA and BHT.

Peanut Oil was pressed from unroasted peanuts after the skins were removed. Each test compound was spiked into the oil at two levels, ~ 100 ppm and ~ 20 ppm, with the actual levels given in Table 7. 50 μ L of methanol solubilized antioxidant was added to each sample to aid in dispersion of the antioxidant. A control

sample was prepared with 50 μ L of methanol containing no antioxidant.

The samples were evaluated in duplicate, for oxidative stability using the Rancimat stability test at 5 100°C and 20 cc/min of air. Experimental parameters were chosen to match those used with the Active Oxygen Method (AOM) or Swift Stability Test (Van Oosten et al., 1981). A typical Rancimat trace is shown in Figure 16. Results are reported in Table 8 as hours required to reach a 10 peroxide level of 100 meq.

Table 7: Concentrations of Antioxidants

	SAMPLE	LEVEL 1	LEVEL 2
		ppm	
15	Butylated Hydroxytoluene (BHT)	24	120
	Butylated Hydroxyanisole (BHA)	24	120
20	Crude Ethyl Acetate Fraction of Cocoa	22	110
	Fraction A-C	20	100
	Fraction D-E	20	100

**Table 8: Oxidative Stability of Peanut Oil
with Various Antioxidants**

SAMPLE	20 ppm	100 ppm
	average	
Control	10.5 ± 0.7	
BHT	16.5 ± 2.1	12.5 ± 2.1
BHA	13.5 ± 2.1	14.0 ± 1.4
Crude Cocoa Fraction	18.0 ± 0.0	19.0 ± 1.4
Fraction A-C	16.0 ± 6.4	17.5 ± 0.0
Fraction D-E	14.0 ± 1.4	12.5 ± 0.7

10 These results demonstrated increased oxidative stability of peanut oil with all of the additives tested. The highest increase in oxidative stability was realized by the sample spiked with the crude ethyl acetate extract of cocoa. These results demonstrated that cocoa extracts 15 containing procyanidins have antioxidant potential equal to or greater than equal amounts of synthetic BHA and BHT. Accordingly, the invention may be employed in place of BHT or BHA in known utilities of BHA or BHT, for instance as an antioxidant and/or food additive. And, in 20 this regard, it is noted too that the invention is from an edible source. Given these results, the skilled artisan can also readily determine a suitable amount of the invention to employ in such "BHA or BHT" utilities, e.g., the quantity to add to food, without undue 25 experimentation.

Example 10: Topoisomerase II Inhibition Study

DNA topoisomerase I and II are enzymes that catalyze the breaking and rejoining of DNA strands, thereby controlling the topological states of DNA (Wang, 30 1985). In addition to the study of the intracellular

function of topoisomerase, one of the most significant findings has been the identification of topoisomerase II as the primary cellular target for a number of clinically important antitumor compounds (Yamashita et al., 1990) which include intercalating agents (*m*-AMSA, Adriamycin® and ellipticine) as well as nonintercalating epipodophyllotoxins. Several lines of evidence indicate that some antitumor drugs have the common property of stabilizing the DNA - topoisomerase II complex ("cleavable complex") which upon exposure to denaturing agents results in the induction of DNA cleavage (Muller et al., 1989). It has been suggested that the cleavable complex formation by antitumor drugs produces bulky DNA adducts that can lead to cell death.

According to this attractive model, a specific new inducer of DNA topoisomerase II cleavable complex is useful as an anti-cancer, anti-tumor or antineoplastic agent. In an attempt to identify cytotoxic compounds with activities that target DNA, the cocoa procyanidins were screened for enhanced cytotoxic activity against several DNA - damage sensitive cell lines and enzyme assay with human topoisomerase II obtained from lymphoma.

A. Decatenation of Kinetoplast DNA by Topoisomerase II

The *in vitro* inhibition of topoisomerase II decatenation of kinetoplast DNA, as described by Muller et al. (1989), was performed as follows. Nuclear extracts containing topoisomerase II activity were prepared from human lymphoma by modifications of the methods of Miller et al. (1981) and Danks et al. (1988). One unit of purified enzyme was enough to decatenate 0.25 µg of kinetoplast DNA in 30 min. at 34°C. Kinetoplast DNA was obtained from the trypanosome *Crithidia fasciculata*. Each reaction was carried out in a 0.5mL microcentrifuge tube containing 19.5µL H₂O, 2.5µL 10X buffer (1X buffer contains 50mM tris-HCl, pH 8.0, 120mM KCl, 10mM MgCl₂, 0.5mM ATP, 0.5mM dithiothreitol and 30µg

BSA/mL), 1 μ L kinetoplast DNA (0.2 μ g), and 1 μ L DMSO-containing cocoa procyanidin test fractions at various concentrations. This combination was mixed thoroughly and kept on ice. One unit of topoisomerase was added 5 immediately before incubation in a waterbath at 34°C for 30 min.

Following incubation, the decatenation assay was stopped by the addition of 5 μ L stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and 10 placed on ice. DNA was electrophoresed on a 1% agarose gel in TAE buffer containing ethidium bromide (0.5 μ g/mL). Ultraviolet illumination at 310nm wavelength allowed the visualization of DNA. The gels were photographed using a Polaroid Land camera.

15 Figure 17 shows the results of these experiments. Fully catenated kinetoplast DNA does not migrate into a 1% agarose gel. Decatenation of kinetoplast DNA by topoisomerase II generates bands of monomeric DNA (monomer circle, forms I and II) which do 20 migrate into the gel. Inhibition of the enzyme by addition of cocoa procyanidins is apparent by the progressive disappearance of the monomer bands as a function of increasing concentration. Based on these results, cocoa procyanidin fractions A, B, D, and E were 25 shown to inhibit topoisomerase II at concentrations ranging from 0.5 to 5.0 μ g/mL. These inhibitor concentrations were very similar to those obtained for mitoxanthrone and m-AMSA (4'-(9-acridinylamino)methanesulfon-m-anisidine).

30 **B. Drug Sensitive Cell Lines**

Cocoa procyanidins were screened for cytotoxicity against several DNA-damage sensitive cell lines. One of the cell lines was the xrs-6 DNA double strand break repair mutant developed by P. Jeggo (Kemp et 35 al., 1984). The DNA repair deficiency of the xrs-6 cell line renders them particularly sensitive to x-irradiation, to compounds that produce DNA double strand

breaks directly, such as bleomycin, and to compounds that inhibit topoisomerase II, and thus may indirectly induce double strand breaks as suggested by Warters et al. (1991). The cytotoxicity toward the repair deficient 5 line was compared to the cytotoxicity against a DNA repair proficient CHO line, BR1. Enhanced cytotoxicity towards the repair deficient (xrs-6) line was interpreted as evidence for DNA cleavable double strand break formation.

10 The DNA repair competent CHO line, BR1, was developed by Barrows et al. (1987) and expresses O⁶-alkylguanine - DNA - alkyltransferase in addition to normal CHO DNA repair enzymes. The CHO double strand break repair deficient line (xrs-6) was a generous gift 15 from Dr. P. Jeggo and co-workers (Jeggo et al., 1989). Both of these lines were grown as monolayers in alpha-MEM containing serum and antibiotics as described in Example 6. Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. Before treatment with cocoa 20 procyanidins, cells grown as monolayers were detached with trypsin treatment. Assays were performed using the MTT assay procedure described in Example 6.

The results (Figure 18) indicated no enhanced cytotoxicity towards the xrs-6 cells suggesting that the 25 cocoa procyanidins inhibited topoisomerase II in a manner different from cleavable double strand break formation. That is, the cocoa procyanidins interact with topoisomerase II before it has interacted with the DNA to form a noncleavable complex.

30 Noncleavable complex forming compounds are relatively new discoveries. Members of the anthracyclines, podophyllin alkaloids, anthracenediones, acridines, and ellipticines are all approved for clinical anti-cancer, anti-tumor or antineoplastic use, and they 35 produce cleavable complexes (Liu, 1989). Several new classes of topoisomerase II inhibitors have recently been identified which do not appear to produce cleavable

complexes. These include amonafide (Hsiang et al., 1989), distamycin (Fesen et al., 1989), flavanoids (Yamashita et al., 1990), saintopin (Yamashita et al., 1991), membranone (Drake et al., 1989), terpenoids 5 (Kawada et al., 1991), anthrapyrazoles (Fry et al., 1985), dioxopiperazines (Tanabe et al., 1991), and the marine acridine - dercitin (Burres et al., 1989).

Since the cocoa procyanidins inactivate topoisomerase II before cleavable complexes are formed, 10 they have chemotherapy value either alone or in combination with other known and mechanistically defined topoisomerase II inhibitors. Additionally, cocoa procyanidins also appear to be a novel class of topoisomerase II inhibitors, (Kashiwada et al., 1993) and 15 may thus be less toxic to cells than other known inhibitors, thereby enhancing their utility in chemotherapy.

The human breast cancer cell line MCF-7 (ADR) which expresses a membrane bound glycoprotein (gp170) to 20 confer multi-drug resistance (Leonessa et al., 1994) and its parental line MCF-7 p168 were used to assay the effects of cocoa fraction D & E. As shown in Figure 19, the parental line was inhibited at increasing dose levels of fraction D & E, whereas the Adriamycin (ADR) resistant 25 line was less effected at the higher doses. These results show that cocoa fraction D & E has an effect on multi-drug resistant cell lines.

Example 11: Synthesis of Procyanidins

The synthesis of procyanidins was performed 30 according to the procedures developed by Delcour et al. (1983), with modification. In addition to condensing (+)-catechin with dihydroquercetin under reducing conditions, (-)-epicatechin was also used to reflect the high concentrations of (-)-epicatechin that naturally 35 occur in unfermented cocoa beans. The synthesis products were isolated, purified, analyzed, and identified by the procedures described in Examples 3, 4 and 5. In this

manner, the biflavanoids, triflavanoids and tetraflavanoids are prepared and used as analytical standards and, in the manner described above with respect to cocoa extracts.

5 Example 12: Assay of Normal Phase Semi-Preparative Fractions

Since the polyphenol extracts are compositionally complex, it was necessary to determine which components were active against cancer cell lines 10 for further purification, dose-response assays and comprehensive structural identification. A normal phase semi preparative HPLC separation (Example 3B) was used to separate cocoa procyanidins on the basis of oligomeric size. In addition to the original extract, twelve 15 fractions were prepared (Figures 2B and 15 O) and assayed at 100 μ g/mL and 25 μ g/mL doses against HeLa and SKBR-3 cancer cell lines to determine which oligomer possessed the greatest activity. As shown in Figures 20A and B, fractions 4-11 (pentamer-dodecamer) significantly 20 inhibited HeLa and SKBr-3 cancer cell lines at the 100 μ g/mL level. These results indicated that these specific oligomers had the greatest activity against HeLa and SKBR-3 cells. Additionally, normal phase HPLC analysis of cocoa fraction D & E indicated that this 25 fraction, used in previous investigations, e.g., Example 7, was enriched with these oligomers.

Example 13: HPLC Purification Methods

Method A. GPC Purification

Procyanidins obtained as in Example 2 were 30 partially purified by liquid chromatography on Sephadex LH 20 (72.5 x 2.5cm), using 100% methanol as the eluting solvent, at a flow rate of 3.5mL/min. Fractions of the eluent were collected after the first 1.5 hours, and the fractions were concentrated by a rotary evaporator, 35 redissolved in water and freeze dried. These fractions were referred to as pentamer enriched fractions. Approximately 2.00g of the extract obtained from Example

2 was subfractionated in this manner. Results are shown in Table 9.

Table 9: Composition of Fractions Obtained:

Fraction (Time)	Monomer (% Area)	Dimer (% Area)	Trimer (% Area)	Tetramer (% Area)	Pentamer (% Area)	Hexamer (% Area)	Heptamer (% Area)	Octamer (% Area)	Nonamer (% Area)	Decamer (% Area)	Undecamer (% Area)	Others (% Area)
1:15	73	8	16	3	ND	ND	ND	ND	ND	ND	ND	ND
1:44	67	19	10	3	1	tr	tr	tr	tr	tr	tr	tr
2:13	30	29	24	11	4	1	tr	tr	tr	tr	tr	tr
2:42	2	16	31	28	15	6	2	tr	tr	tr	tr	tr
3:11	1	12	17	25	22	13	7	.2	1	tr	tr	tr
3:40	tr	18	13	18	20	15	10	5	2	tr	tr	tr
4:09	tr	6	8	17	21	19	14	8	4	2	tr	tr

ND = not detected
tr = trace amount

Method B. Normal Phase Separation

Procyanidins obtained as Example 2 were separated purified by normal phase chromatography on Supelcosil LC-Si, 100Å, 5µm (250 x 4.6mm), at a flow rate 5 of 1.0mL/min, or, in the alternative, Lichrosphere® Silica 100, 100Å, 5µm (235 x 3.2mm), at a flow rate of 0.5mL/min. Separations were aided by a step gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86). Mobile phase composition was A = dichloromethane; B = methanol; and C = acetic acid:water (1:1). Components were detected by fluorescence where λ_{ex} = 276nm and λ_{em} = 316nm, and by UV at 280nm. The injection volume was 5.0µL (20mg/mL) of the procyanidins 15 obtained from Example 2. These results are shown in Fig. 40A and 40B.

In the alternative, separations were aided by a step gradient under the following conditions: (Time, %A, %B); (0, 76, 20); (25, 46, 50); (30, 10, 86). Mobile 20 phase composition was A = dichloromethane; B = methanol; and C = acetic acid : water (1:1). The results are shown in Fig. 41A and 41B.

Method C. Reverse - Phase Separation

Procyanidins obtained as in Example 2 were 25 separated purified by reverse phase chromatography on Hewlett Packard Hypersil ODS 5µm. (200 x 2.1mm), and a Hewlett Packard Hypersil ODS 5µm guard column (20 x 2.1mm). The procyanidins were eluted with a linear gradient of 20% B into A in 20 minutes, followed by a 30 column wash with 100% B at a flow rate of 0.3mL/min. The mobile phase composition was a degassed mixture of B = 1.0% acetic acid in methanol and A = 2.0% acetic acid in nanopure water. Components were detected by UV at 280nm, and fluorescence where λ_{ex} = 276nm and λ_{em} = 316nm; and 35 the injection volume was 2.0µL (20mg/mL).

Example 14: HPLC Separation of Pentamer Enriched Fractions

Method A. Semi-Preparative Normal Phase HPLC

The pentamer enriched fractions were further purified by semi-preparative normal phase HPLC by a Hewlett Packard 1050 HPLC system equipped with a 5 Millipore - Waters model 480 LC detector set at 254nm, which was assembled with a Pharmacia Frac-100 Fraction Collector set to peak mode. Separations were effected on a Supelco 5 μ m Supelcosel LC-Si, 100 \AA column (250 x 10mm) connected with a Supelco 5 μ Supelguard LC-Si guard column (20 x 4.6mm). Procyanidins were eluted by a linear gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by a 10 minute re-equilibration. Mobile phase composition was A = dichloromethane; B = methanol; and C = acetic acid:water (1:1). A flow rate of 3mL/min was used. Components were detected by UV at 254nm; and recorded on a Kipp & Zonan BD41 recorder. Injection volumes ranged from 100-250 μ l of 10mg of procyanidin extracts dissolved in 0.25mL 70% aqueous acetone. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further purification and subsequent evaluation.

HPLC conditions: 250 x 100mm Supelco Supelcosil LC-Si
25 (5 μ m) Semipreparative Column
20 x 4.6mm Supelco Supelcosil LC-Si
(5 μ m) Guard Column
Detector: Waters LC
Spectrophotometer Model
30 480 @ 254nm
Flow rate: 3mL/min.,
Column Temperature: ambient,
Injection: 250 μ L of pentamer enriched
extract

35

acetic acid:
Gradient: CH₂Cl₂ methanol water (1:1)

0	82	14	4
30	67.6	28.4	4
60	46	50	4
65	10	86	4
5	70	10	86

Method B. Reverse Phase Separation

Procyanidin extracts obtained as in Example 13
 10 were filtered through a 0.45μ nylon filter and analyzed by a Hewlett Packard 1090 ternary phase HPLC system equipped with a Diode Array detector and a HP model 1046A Programmable Fluorescence Detector. Separations were effected at 45°C on a Hewlett Packard 5μ Hypersil ODS
 15 column ($200 \times 2.1\text{mm}$). The procyanidins were eluted with a linear gradient of 60% B into A followed by a column wash with B at a flow rate of 0.3mL/min . The mobile phase composition was a de-gassed mixture of B = 0.5% acetic acid in methanol and A = 0.5% acetic acid in
 20 nanopure water. Acetic acid levels in A and B mobile phases can be increased to 2%. Components were detected by fluorescence, where $\lambda_{\text{ex}} = 276\text{nm}$ and $\lambda_{\text{em}} = 316\text{nm}$, and by UV at 280nm . Concentrations of (+)-catechin and (-)-epicatechin were determined relative to reference
 25 standard solutions. Procyanidin levels were estimated by using the response factor for (-)-epicatechin.

Method C. Normal Phase Separation

Pentamer enriched procyanidin extracts obtained as in Example 13 were filtered through a 0.45μ nylon
 30 filter and analyzed by a Hewlett Packard 1090 Series II HPLC system equipped with a HP Model 1046A Programmable Fluorescence detector and Diode Array detector. Separations were effected at 37°C on a 5μ Phenomenex Lichrosphere[®] Silica 100 column ($250 \times 3.2\text{mm}$) connected
 35 to a Supelco Supelguard LC-Si 5μ guard column ($20 \times 4.6\text{mm}$). Procyanidins were eluted by linear gradient under the following conditions: (time, %A, %B); (0, 82,

14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86), followed by an 8 minute re-equilibration.

Mobile phase composition was A = dichloromethane, B = methanol, and C = acetic acid:water at a volume ratio of 5 1:1. A flow rate of 0.5mL/min was used. Components were detected by fluorescence, where $\lambda_{ex} = 276\text{nm}$ and $\lambda_{em} = 316\text{nm}$ or by UV at 280nm. A representative HPLC chromatogram showing the separation of the various procyanidins is shown in Figure 2 for one genotype.

10 Similar HPLC profiles were obtained from other *Theobroma*, *Herrania* and/or their inter or intra specific crosses.

HPLC conditions:

250 x 3.2mm Phenomenex Lichrosphere[®] Silica 100 column (5 μ) 20 x 4.6mm Supelco Supelguard LC-Si (5 μ)

15 guard column

Detectors: Photodiode Array @ 280nm

Fluorescence $\lambda_{ex} = 276\text{nm}$; $\lambda_{em} = 316\text{nm}$

Flow rate: 0.5 mL/min.

Column temperature: 37°C

20 acetic acid:

<u>Gradient:</u>	<u>CH₂Cl₂</u>	<u>methanol</u>	<u>water (1:1)</u>
0	82	14	4
30	67.6	28.4	4
25	60	50	4
	65	86	4
	70	86	4

Method D. Preparative Normal Phase Separation

30 The pentamer enriched fractions obtained as in Example 13 were further purified by preparative normal phase chromatography by modifying the method of Rigaud et al., (1993) J. Chrom. 654, 255-260.

Separations were affected at ambient 35 temperature on a 5 μ Supelcosil LC-Si 100Å column (50 x

2cm), with an appropriate guard column. Procyanidins were eluted by a linear gradient under the following conditions: (time, %A, %B, flow rate); (0, 92.5, 7.5, 10); (10, 92.5, 7.5, 40); (30, 91.5, 18.5, 40); (145, 88, 5 22, 40); (150, 24, 86, 40); (155, 24, 86, 50); (180, 0, 100, 50). Prior to use, the mobile phase components were mixed by the following protocol:

Solvent A preparation (82% CH₂Cl₂, 14% methanol, 2% acetic acid, 2% water):

10 1. Measure 80mL of water and dispense into a 4L bottle.

2. Measure 80mL of acetic acid and dispense into the same 4L bottle.

15 3. Measure 560mL of methanol and dispense into the same 4L bottle.

4. Measure 3280mL of methylene chloride and dispense into the 4L bottle.

5. Cap the bottle and mix well.

20 6. Purge the mixture with high purity Helium for 5-10 minutes to degas.

Repeat steps 1-6 two times to yield 8 volumes of solvent A.

Solvent B preparation (96% methanol, 2% acetic acid, 2% water):

25 1. Measure 80mL of water and dispense into a 4L bottle.

2. Measure 80mL of acetic acid and dispense into the same 4L bottle.

3. Measure 3840mL of methanol and dispense
3840mL of methanol and dispense into the same 4L bottle.

4. Cap the bottle and mix well.

5. Purge the mixture with high purity Helium
5 for 5-10 minutes to degas.

Repeat steps 1-5 to yield 4 volumes of solvent

B. Mobile phase composition was A = methylene chloride
with 2% acetic acid and 2% water; B = methanol with 2%
acetic acid and 2% water. The column load was 0.7g in
10 7mL. components were detected by UV at 254nm. A typical
preparative normal phase HPLC separation of cocoa
procyanidins is shown in Figure 42.

HPLC Conditions:

Column: 50 x 2cm 5 μ Supelcosil LC-Si run @
15 ambient temperature.

Mobile Phase: A = Methylene Chloride with 2%
Acetic Acid and 2% Water.

20 B = Methanol with 2% Acetic Acid
and 2% Water.

Gradient/Flow Profile:

	TIME (MIN)	%A	%B	FLOW RATE (mL/min)
5	0	92.5	7.5	10
	10	92.5	7.5	40
	30	91.5	8.5	40
	145	88.0	22.0	40
	150	24.0	86.0	40
10	155	24.0	86.0	50
	180	0.0	100.0	50

Example 15: Identification of Procyanidins

15 Procyanidins obtained as in Example 14, method D were analyzed by Matrix Assisted Laser Desorption Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF/MS) using a HP G2025A MALDI-TOF/MS system equipped with a Lecroy 9350 500 MHz Oscilloscope. The instrument 20 was calibrated in accordance with the manufacturer's instructions with a low molecular weight peptide standard (HP Part No. G2051A) or peptide standard (HP Part No. G2052A) with 2,5-dihydroxybenzoic acid (DHB) (HP Part No. G2056A) as the sample matrix. One (1.0) mg of sample was 25 dissolved in 500 μ L of 70/30 methanol/water, and the sample was then mixed with DHB matrix, at a ratio of 1:1, 1:10 or 1:50 (sample:matrix) and dried on a mesa under vacuum. The samples were analyzed in the positive ion mode with the detector voltage set at 4.75kV and the 30 laser power set between 1.5 and 8 μ J. Data was collected as the sum of a number of single shots and displayed as units of molecular weight and time of flight. A representative MALDI-TOF/MS is shown in Figure 22A.

Figures 22 and C show MALDI-TOF/MS spectra obtained from partially purified procyanidins prepared as described in Example 3, Method A and used for *in vitro* assessment as described in Examples 6 and 7, and whose 5 results are summarized in Table 6. This data illustrates that the inventive compounds described herein were predominantly found in fractions D-E, but not A-C.

The spectra were obtained as follows:

The purified D-E fraction was subjected to 10 MALDI-TOF/MS as described above, with the exception that the fraction was initially purified by SEP-PACK® C-18 cartridge. Five (5) mg of fraction D-E in 1 mL nanopure water was loaded onto a pre-equilibrated SEP-PACK® cartridge. The column was washed with 5mL nanopure water 15 to eliminate contaminants, and procyanidins were eluted with 1mL 20% methanol. Fractions A-C were used directly, as they were isolated in Example 3, Method A, without further purification.

These results confirmed and extended earlier 20 results (see Example 5, Table 3, Figs. 20A and B) and indicate that the inventive compounds have utility as sequestrants of cations. In particular, MALDI-TOF/MS results conclusively indicated that procyanidin oligomers of n = 5 and higher (see Figures 20A and B; and formula 25 under Objects and Summary of the Invention) were strongly associated with anti-cancer activity with the HeLa and SKBR-3 cancer cell line model. Oligomers of n = 4 or less were ineffective with these models. The pentamer structure apparently has a structural motif which is 30 present in it and in higher oligomers which provides the activity. Additionally, it was observed that the MALDI-TOF/MS data showed strong M⁺ ions of Na⁺, 2 Na⁺, K⁺, 2 K⁺, Ca⁺⁺, demonstrating the utility as cation sequestrants.

Example 16: Purification of Oligomeric Fractions
35 Method A. Purification by Semi-
Preparative Reverse Phase HPLC

Procyanidins obtained from Example 14, Method A and B and D were further separated to obtain experimental quantities of like oligomers for further structural identification and elucidation (e.g., Example 15, 18, 19, 5 and 20). A Hewlett Packard 1050 HPLC system equipped with a variable wavelength detector, Rheodyne 7010 injection valve with 1mL injection loop was assembled with a Pharmacia FRAC-100 Fraction Collector.

Separations were effected on a Phenomenex Ultracarb[®] 10 μ 10 ODS column (250 x 22.5mm) connected with a Phenomenex 10 μ ODS Ultracarb[®] (60 x 10mm) guard column. The mobile phase composition was A = water; B = methanol used under the following linear gradient conditions: (time, %A); (0, 85), (60, 50), (90, 0 and (110, 0) at a flow rate of 5 15 mL/min. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further evaluation by MALDI-TOF/MS and NMR. Injection loads ranged from 25-100mg of material. A representative elution profile is shown in 20 Fig. 23b.

Method B. Modified Semi-Preparative HPLC

Procyanidins obtained from Example 14, Method A and B and D were further separated to obtain experimental quantities of like oligomers for further structural 25 identification and elucidation (e.g., Example 15, 18, 19, and 20). Supelcosil LC-Si 5 μ column (250 x 10mm) with a Supelcosil LC-Si 5 μ (20 x 2mm) guard column. The separations were effected at a flow rate of 3.0mL/min, at ambient temperature. The mobile phase composition was 30 A = dichloromethane; B = methanol; and C = acetic acid:water (1:1); used under the following linear gradient conditions: (time, %A, %B); (0, 82, 14); (22, 74, 21); (32, 74, 21); (60, 74, 50, 4); (61, 82, 14), followed by column re-equilibration for 7 minutes. 35 Injection volumes were 60 μ L containing 12mg of enriched pentamer. Components were detected by UV at 280nm. A representative elution profile is shown in Figure 23A.

Example 17: Molecular Modeling of Pentamers

Energy minimized structures were determined by molecular modeling using Desktop Molecular Modeller, version 3.0, Oxford University Press, 1994. Four representative views of $[EC(4 \rightarrow 8)]_4\text{-EC}$ (EC = epicatechin) pentamers based on the structure of epicatechin are shown in Figures 24 A-D. A helical structure is suggested. In general when epicatechin is the first monomer and the bonding is 4 \rightarrow 8, a beta configuration results, when the first monomer is catechin and the bonding is 4 \rightarrow 8, an alpha configuration results; and, these results are obtained regardless of whether the second monomer is epicatechin or catechin (an exception is ent-EC(4 \rightarrow 8)ent-EC). Figures 38A - 38P show preferred pentamers, and, Figures 39A to 39P show a library of stereoisomers up to and including the pentamer, from which other compounds within the scope of the invention can be prepared, without undue experimentation.

Example 18: NMR Evaluation of Pyrocyanidins

^{13}C NMR spectroscopy was deemed a generally useful technique for the study of procyanidins, especially as the phenols usually provide good quality spectra, whereas proton NMR spectra are considerably broadened. The ^{13}C NMR spectra of oligomers yielded useful information for A or B ring substitution patterns, the relative stereochemistry of the C ring and in certain cases, the position of the interflavanoid linkages. Nonetheless, ^1H NMR spectra yielded useful information.

Further, HOHAHA, makes use of the pulse technique to transfer magnetization of a first hydrogen to a second in a sequence to obtain cross peaks corresponding to alpha, beta, gamma or delta protons. COSY is a 2D-Fourier transform NMR technique wherein vertical and horizontal axes provide ^1H chemical shift and 1D spectra; and a point of intersection provides a correlation between protons, whereby spin-spin couplings

can be determined. HMQC spectra enhances the sensitivity of NMR spectra of nuclei; other than protons and can reveal cross peaks from secondary and tertiary carbons to the respective protons. APT is a ^{13}C technique used in 5 determining the number of hydrogens present at a carbon. An even number of protons at a carbon will result in a positive signal, while an odd number of protons at a carbon will result in a negative signal.

Thus ^{13}C NMR, ^1H NMR, HOHAHA (homonuclear 10 Hartmann-Hahn), HMQC (heteronuclear multiple quantum coherence), COSY (Homonuclear correlation spectroscopy), APT (attached proton test), and XHCORR (a variation on HMQC) spectroscopy were used to elucidate the structures of the inventive compounds.

15 Method A. Monomer

All spectra were taken in deuterated methanol, at room temperature, at an approximate sample concentration of 10mg/mL. Spectra were taken on a Bruker 500 MHZ NMR, using methanol as an internal standard.

20 Figures 44A-E represent the NMR spectra which were used to characterize the structure of the epicatechin monomer. Figure 44A shows the ^1H and ^{13}C chemical shifts, in tabular form. Figures 44 B-E show ^1H , APT, XHCORR and COSY spectra for epicatechin.

25 Similarly, Figures 45A-F represent the NMR spectra which were used to characterize the structure of the catechin monomer. Figure 45A shows the ^1H and ^{13}C chemical shifts, in tabular form. Figures 44 B-F show ^1H , ^{13}C , APT, XHCORR and COSY spectra for catechin.

30 Method B. Dimers

All spectra were taken in 75% deuterated acetone in D_2O , using acetone as an internal standard, and an approximate sample concentration of 10mg/mL.

35 Figures 46A-G represent the spectra which were used to characterize the structure of the B2 dimer. Fig. 46A shows ^1H and ^{13}C chemical shifts, in tabular form.

100

The terms T and B indicate the top half of the dimer and the bottom half of the dimer.

Figures 46B and C show the ^{13}C and APT spectra, respectively, taken on a Bruker 500 MHZ NMR, at room
5 temperature.

Figures 46D-G show the ^1H , HMQC, COSY and HOHAHA, respectively, which were taken on AMX-360 MHZ NMR at a -7°C. The COSY spectrum was taken using a gradient pulse.

10 Figures 47A-G represent the spectra which were used to characterize the structure of the B5 dimer. Figure 47A shows the ^{13}C and ^1H chemical shifts, in tabular form.

15 Figures 47B-D show the ^1H , ^{13}C and APT, respectively, which were taken on a Bruker 500 MHZ NMR, at room temperature.

Figure 47E shows the COSY spectrum, taken on an AMX-360, at room temperature, using a gradient pulse.

20 Figures 47F and G show the HMQC and HOHAHA, respectively, taken on an AMX-360 MHZ NMR, at room temperature.

Method C. Trimer - Epicatechin/Catechin

All spectra were taken in 75% deuterated acetone in D_2O , at -3°C using acetone as an internal standard, on an AMX-360 MHZ NMR, and an appropriate sample concentration of 10mg/mL.

25 Figures 48A-D represent the spectra which were used to characterize the structure of the epicatechin/catechin trimer. These figures show ^1H , COSY, HMQC and HOHAHA, respectively. The COSY spectrum was taken using a gradient pulse.

Method D. Trimer -All Epicatechin

30 All spectra were taken in 70% deuterated acetone in D_2O , at -1.8°C, using acetone as an internal standard, on an AMX-360 MHZ NMR, and an appropriate sample concentration of 10mg/mL.

Figures 49A-D represent the spectra which were used to characterize the structure of all epicatechin trimer. These figures show ^1H , COSY, HMQC and HOHAHA, respectively. The COSY spectrum was taken using a 5 gradient pulse.

Example 19: Thiolysis of Procyanidins

In an effort to characterize the structure of procyanidins, benzyl mercaptan (BM) was reacted with catechin, epicatechin or dimers B2 and B5. Benzyl 10 mercaptan, as well as phloroglucinol and thiophenol, can be utilized in the hydrolysis (thiolytic) of procyanidins in an alcohol/acetic acid environment. Catechin, epicatechin or dimer (1:1 mixture of B2 and B5 15 dimers) (2.5mg) was dissolved in 1.5mL ethanol, 100 μL BM and 50 μL acetic acid, and the vessel (Beckman amino acid analysis vessel) was evacuated and purged with nitrogen repeatedly until a final purge with nitrogen was followed by sealing the reaction vessel. The reaction vessel was placed in a heat block at 95°C, and aliquots of the 20 reaction were taken at 30, 60, 120 and 240 minutes. The relative fluorescence of each aliquot is shown in Figures 25A-C, representing epicatechin, catechin and dimers, respectively. Higher oligomers are similarly thiolyzed.

Example 20: Thiolytic and Desulfurization of Dimers

Dimers B2 and B5 were hydrolyzed with 25 benzylmercaptan by dissolving dimer (B2 or B5; 1.0 mg) in 600 μl ethanol, 40 μl BM and 20 μl acetic acid. The mixture was heated at 95°C for 4 hours under nitrogen in a Beckman Amino Acid Analysis vessel. Aliquots were 30 removed for analysis by reverse-phase HPLC, and 75 μl of each of ethanol Raney Nickel and gallic acid (10mg/mL) were added to the remaining reaction medium in a 2mL hypovial. The vessel was purged under hydrogen, and occasionally shaken for 1 hour. The product was filtered 35 through a 0.45 μ filter and analyzed by reverse-phase HPLC. Representative elution profiles are shown in Figures 26 A and B. Higher oligomers are similarly

desulfurized. This data suggests polymerization of epicatechan or catechin and therefore represents a synthetic route for preparation of inventive compounds.

Example 21: In vivo Activity of Pentamer in MDA MB 231

5 Nude Mouse Model

MDA-MB-231/LCC6 cell line. The cell line was grown in improved minimal essential medium (IMEM) containing 10% fetal bovine serum and maintained in a
10 humidified, 5% CO₂ atmosphere at 37°C.

Mice. Female six to eight week old NCr nu/nu (athymic) mice were purchased through NCI and housed in an animal facility and maintained according to the regulations set forth by the United States Department of
15 Agriculture, and the American Association for the Accreditation of Laboratory Animal Care. Mice with tumors were weighed every other day, as well as weekly to determine appropriate drug dosing.

Tumor implantation. MDA-MD-231 prepared by
20 tissue culture was diluted with IMEM to 3.3 x 10⁶ cells/mL and 0.15mL (i.e. 0.5 x 10⁶ cells) were injected subcutaneously between nipples 2 and 3 on each side of the mouse. Tumor volume was calculated by multiplying:
length x width x height x 0.5. Tumor volumes over a
25 treatment group were averaged and Student's t test was used to calculate p values.

Sample preparation. Plasma samples were obtained by cardiac puncture and stored at -70°C with 15-
30 20 mM EDTA for the purposes of blood chemistry determinations. No differences were noted between the control group and experimental groups.

Fifteen nude mice previously infected with 500,000 cells subcutaneously with tumor cell line MDA-MB-231, were randomly separated into three groups of 5 animals each and treated by intraperitoneal injection with one of: (i) placebo containing vehicle alone (DMSO); (ii) 2mg/mouse of purified pentameric procyanidin

extract as isolated in Example 14 method D in vehicle (DMSO); and (iii) 10mg/mouse purified pentameric procyanidin extract as isolated in Example 14, method D in vehicle (DMSO).

5 The group (iii) mice died within approximately 48 to 72 hours after administration of the 10mg, whereas the group (ii) mice appeared normal. The cause of death of the group (iii) mice was undetermined; and, cannot necessarily be attributed to the administration of
10 inventive compounds. Nonetheless, 10mg was considered an upper limit with respect to toxicity.

15 Treatment of groups (i) and (ii) was repeated once a week, and tumor growth was monitored for each experimental and control group. After two weeks of treatment, no signs of toxicity were observed in the mice of group (ii) and, the dose administered to this group was incrementally increased by 1/2 log scale each subsequent week. The following Table represents the dosages administered during the treatment schedule for
20 mice of group (ii):

	Week	Dose
		(mg/mouse)
25	1	2
	2	2
	3	4
	4	5
	5	5
	6	5
	7	5

30

The results of treatment are shown in Figures 27A and B and Table 10.

TABLE 10: IN VIVO ANTI-CANCER RESULTS

DAY	% SURVIVAL GROUP (i)	% SURVIVAL GROUP (ii)	% SURVIVAL GROUP (iii)
5	1	100	100
	2	100	100
	3	100	100
	4	100	100
	5	100	100
10	6	100	100
	7	100	100
	8	100	100
	9	100	100
	10	100	100
15	11	100	100
	12	100	100
	13	100	100
	14	100	100
	15	100	100
20	16	100	100
	17	100	100
	18	100	100
	19	100	100
	20	100	100
25	21	100	100
	22	75	100

DAY	% SURVIVAL GROUP (i)	% SURVIVAL GROUP (ii)	% SURVIVAL GROUP (iii)
5	23	75	100
	24	75	100
	25	75	100
	26	75	100
	27	75	100
	28	75	100
	29	50	100
	30	50	100
	31	50	100
	32	50	100
10	33	50	100
	34	50	100
	35	50	100
	36	25	100
	37	25	100
15	38	25	100
	39	25	100
	40	25	100
	41	25	100
	42	25	100
20	43	25	80
	44	25	80
	45	25	80
	46	25	80

DAY	% SURVIVAL GROUP (i)	% SURVIVAL GROUP (ii)	% SURVIVAL GROUP (iii)
5	47	25	80
	48	25	80
	49	25	80
	50	25	60
	51	25	60
	52	25	60
	53	25	60
	54	25	60
	55	25	60
	56	25	60
10	57	0	40
	58		40
	59		40
	60		40
	61		40
15	62		40
	63		40
	64		40

20 These results demonstrate that the inventive fractions and the inventive compounds indeed have utility in antineoplastic compositions, and are not toxic in low to medium dosages, with toxicity in higher dosages able to be determined without undue experimentation.

25 Example 22: Antimicrobial Activity of Cocoa Extracts

Method A:

A study was conducted to evaluate the antimicrobial activity of crude procyanidin extracts from cocoa beans against a variety of microorganisms important in food spoilage or pathogenesis. The cocoa extracts 5 from Example 2, method A were used in the study. An agar medium appropriate for the growth of each test culture (99mL) was seeded with 1 mL of each cell culture suspension in 0.45% saline (final population 10^2 - 10^4 cfu/mL), and poured into petri dishes. Wells were cut 10 into hardened agar with a #2 cork borer (5mm diameter). The plates were refrigerated at 4°C overnight, to allow for diffusion of the extract into the agar, and subsequently incubated at an appropriate growth temperature for the test organism. The results were as 15 follows:

Sample Zone of Inhibition (mm)

Extract Concentration (mg/mL)	<i>B. sphaericus</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>
0	NI	NI	NI	NI	NI
25	NI	12	NI	11	NI
250	12	20	19	19	11
500	14	21	21	21	13

25

NI = no inhibition

Antimicrobial activity of purified procyanidin extracts from cocoa beans was demonstrated in another 30 study using the well diffusion assay described above (in Method A) with *Staphylococcus aureus* as the test culture. The results were as follows:

cocoa extracts: 10mg/100 µL decaffeinated/
detheobrominated acetone extract
as in Example 13, method A

35

108

- 10mg/100 μ L dimer (99% pure)
as in Example 14, method D
10mg/100 μ L tetramer (95% pure) as in
Example 14, method D
5 10mg/100 μ L hexamer (88% pure) as in
Example 14, method D
10mg/100 μ L
octamer/nonamer (92% pure) as in Example 14, method D
10 10mg/100 μ L nonamer & higher (87%
pure) as in Example 14, method D

Sample Zone of Inhibition (mm)

15	0.45% saline	0
	Dimer	33
	Tetramer	27
	Hexamer	24
	0.45% saline	0
20	Octamer	22
	Nonamer	20
	Decaff./detheo.	26

Method B:

- 25 crude procyanidin extract as in Example 2,
method 2 was added in varying concentrations to TSB
(Trypticase Soy Broth) with phenol red (0.08g/L), The TSB
were inoculated with cultures of *Salmonella enteritidis*
30 or *S. newport* (10^5 cfu/mL), and were incubated for 18
hours at 35°C. The results were as follows:

	<i>S. enteritidis</i>	<i>S. Newport</i>
--	-----------------------	-------------------

0mg/mL	+	+
50	+	+
5 100	+	+
250	+	-
500	-	-
750	-	-

10

where + = outgrowth, and - = no growth, as evidenced by the change in broth culture from red to yellow with acid production. Confirmation of inhibition was made by plating from TSB tubes onto XLD plates.

15 This Example demonstrates that the inventive compounds are useful in food preparation and preservation.

This Example further demonstrates that gram negative and gram positive bacterial growth can be inhibited by the inventive compounds. From this, the inventive compounds can be used to inhibit *Helicobacter pylori*. *Helicobacter pylori* has been implicated in causing gastric ulcers and stomach cancer. Accordingly, the inventive compounds can be used to treat or prevent these and other maladies of bacterial origin. Suitable routes of administration, dosages, and formulations can be determined without undue experimentation considering factors well known in the art such as the malady, and the age, weight, sex, general health of the subject.

30 **Example 23: Halogen-free Analytical Separation of Extract**

Procyanidins obtained from Example 2 were partially purified by Analytical Separation by Halogen-free Normal Phase Chromatography on 100Å Supelcosil LC-Si 35 5µm (250 x 4.6mm), at a flow rate of 1.0mL/min, and a column temperature of 37°C. Separations were aided by a linear gradient under the following conditions: (time, %A, %B); (0, 82, 14); (30, 67.6, 28.4); (60, 46, 50).

Mobile phase composition was A = 30/70 % diethyl ether/Toluene; B = Methanol; and C = acetic acid/water (1:1). Components were detected by UV at 280nm. A representative elution profile is shown in Figure 28.

5 **Example 24: Effect of Pore Size of Stationary Phase
for Normal Phase HPLC Separation of
Procyanidins**

To improve the separation of procyandins, the use of a larger pore size of the silica stationary phase 10 was investigated. Separations were effected on Silica-300, 5 μ m, 300 \AA (250 x 2.0mm), or, in the alternative, on Silica-1000, 5 μ m, 1000 \AA (250 x 2.0mm). A linear gradient was employed as mobile phase composition was: A = Dichloromethane; B = Methanol; and C = acetic acid/water 15 (1:1). Components were detected by fluorescence, wherein $\lambda_{\text{ex}} = 276\text{nm}$ and $\lambda_{\text{em}} = 316\text{nm}$, by UV detector at 280nm. The flow rate was 1.0mL/min, and the oven temperature was 37°C. A representative chromatogram from three different columns (100 \AA pore size, from Example 13, Method D) is 20 shown in Figure 29. This shows effective pore size for separation of procyandins.

**Example 25: Obtaining Desired Procyandins Via
Manipulating Fermentation**

25 Microbial strains representative of the succession associated with cocoa fermentation were selected from the M&M/Mars cocoa culture collection. The following isolates were used:

30 *Acetobacter aceti* ATCC 15973
 Lactobacillus sp. (BH 42)
 Candida cruzii (BA 15)
 Saccharomyces cerevisiae (BA 13)
 Bacillus cereus (BE 35)
 Bacillus sphaericus (ME 12)

Each strain was transferred from stock culture to fresh media. The yeasts and *Acetobacter* were incubated 72 hours at 26°C and the bacilli and *Lactobacillus* were incubated 48 hours at 37°C. The slants were harvested 5 with 5mL phosphate buffer prior to use.

Cocoa beans were harvested from fresh pods and the pulp and testa removed. The beans were sterilized with hydrogen peroxide (35%) for 20 seconds, followed by treatment with catalase until cessation of bubbling. The 10 beans were rinsed twice with sterile water and the process repeated. The beans were divided into glass jars and processed according to the regimens detailed in the following Table:

	Water	Ethanol/acid	Fermentation infusate	Model Fermentation
5	daily transfer to fresh water	daily transfer to solutions of alcohol and acid correspondin g to levels determined at each stage of a model pulp fermentation	daily transfer to fermented pulp pasteurized on each successive day of fermentation	bench scale model fermentation in sterile pulp coinoculated with test strains

The bench scale fermentation was performed in duplicate. All treatments were incubated as indicated
10 below:

Day 1: 26°C
 Day 2: 26°C to 50°C
 Day 3: 50°C
 Day 4: 45°C
 15 Day 5: 40°C

The model fermentation was monitored over the duration of the study by plate counts to assess the microbial population and HPLC analysis of the
20 fermentation medium for the production of microbial metabolites. After treatment, the beans were dried under a laminar flow hood to a water activity of 0.64 and were roasted at 66°C for 15 min. Samples were prepared for procyanidin analysis. Three beans per treatment were
25 ground and defatted with hexane, followed by extraction

with an acetone:water:acetic acid (70:29.5:0.5%) solution. The acetone solution extract was filtered into vials and polyphenol levels were quantified by normal phase HPLC as in Example 13, method B. The remaining 5 beans were ground and tasted. The cultural and analytical profiles of the model bench-top fermentation process is shown in Figures 30A - C. The procyanidin profiles of cocoa beans subjected to various fermentation treatments is shown in Figure 30D.

10 This Example demonstrates that the invention need not be limited to any particular cocoa genotype; and, that by manipulating fermentation, the levels of procyanidins produced by a particular *Theobroma* or *Herrania* species or their inter or intra species specific 15 crosses thereof can be modulated, e.g., enhanced.

The following Table shows procyanidin levels determined in specimens which are representative of the *Theobroma* genus and their inter and intra species specific crosses. Samples were prepared as in Examples 1 20 and 2 (methods 1 and 2), and analyzed as in Examples 13, method B. This data illustrates that the extracts containing the inventive compounds are found in *Theobroma* and *Herrania* species, and their intra and inter species specific crosses.

Theobroma and *Hermannia* Species Procyanidin Levels
ppm (ug/g) in defatted powder

SAMPLE	Oligomer										Total
	Monomer	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer	Nonamer	Decamer	
<i>T. grandiflorum</i> x <i>T. cacao</i> 1'	3922	3442	5384	4074	3146	2080	850	421	348	198	11*
<i>T. grandiflorum</i> x <i>T. cacao</i> 2'	3003	4098	5411	3983	2931	1914	1050	577	356	198	11*
<i>T. grandiflorum</i> x <i>T. cacao</i> 3A'	4950	4980	7556	5341	4008	2576	1075	598	301	144	11*
<i>T. grandiflorum</i> x <i>T. cacao</i> 3B'	3880	4498	6488	4930	3706	2560	1208	593	323	174	11*
<i>T. grandiflorum</i> x <i>T. cacao</i> 3C'	3591	5328	4240	3104	2380	1506	815	506	249	11*	24.566
<i>T. grandiflorum</i> x <i>T. cacao</i> 4'	2647										23.194
<i>T. grandiflorum</i> x <i>T. cacao</i> 5'	2754	3855	5299	3872	2994	1990	1158	629	359	196	88
<i>T. grandiflorum</i> x <i>T. cacao</i> 6'	3212	4134	7608	4736	3590	2274	936	446	278	126	ND*
<i>T. grandiflorum</i> x <i>T. cacao</i> 7N'	3652	5683	9512	5358	3858	2454	1207	640	302	144	ND
<i>T. cacao</i> 1'	2608	21178	3090	2704	2241	1586	900	484	301	148	16.240
<i>T. grandiflorum</i> TEFE ²	4773	4096	5259	4748	3804	2444	988	737	335	156	11*
<i>T. grandiflorum</i> TEFE x <i>T. grandiflorum</i> 7	3336	4916	3900	3064	2039	782	435	390	228	ND	23.832
<i>T. grandiflorum</i> x <i>T. subincanum</i> '	4752										23.290
<i>T. cacao</i> SIAL 659, 10	21,929	10,072	10,106	7788	5311	3242	1311	626	422	146	11*
<i>T. speciosum</i> x <i>T. syvestris</i> '	902	346	1350	217	152	120	60	11*	ND	ND	14.519
<i>T. speciosum</i> x <i>T. syvestris</i> '	5694	3250	2766	1490	822	356	141	11*	ND	ND	11*
<i>T. microcarpum</i> ²											60.753
<i>T. cacao</i> , SIAL 659, 10	21,088	9762	9119	7094	4774	2906	1364	608	361	176	11*
<i>T. cacao</i> , SIAL 659, 124	20,887	9892	9474	7337	4906	2929	1334	652	412	302	11*
<i>T. cacao</i> , SIAL 659, 148											27.910
<i>T. cacao</i> , SIAL 659, 196	9552	5780	5062	3360	2140	1160	464	254	138	11*	22.974
<i>T. cacao</i> , SIAL 659, 1120	8581	4685	4070	2527	1628	888	326	166	123	11*	3329
Pod Rec. 10/96, <i>Hermannia</i> mariae	869	1285	545	347	175	97	11*	ND	ND	ND	933
Sample Rec. prior to 10/96, <i>Hermannia</i> mariae	130	354	151	131	116	51	11*	ND	ND	ND	

Example 26: Effect of Procyanidins on NO**Method A.**

5 The purpose of this study is to establish the relationship between procyanidins (as in Example 14, method D) and NO, which is known to induce cerebral vascular dilation. The effects of monomers and higher oligomers, in concentrations ranging from 100 μ g/mL to
10 0.1 μ g/mL, on the production of nitrates (the catabolites of NO), from HUVEC (human umbilical vein endothelial cells) is evaluated. HUVEC (from Clonetics) is investigated in the presence or absence of each procyanidin for 24 to 48 hours. At the end of the
15 experiments, the supernatants are collected and the nitrate content determined by calorimetric assay. In separate experiments, HUVEC is incubated with acetylcholine, which is known to induce NO production, in the presence or absence of procyanidins for 24 to 48
20 hours. At the end of the experiments, the supernatants are collected and nitrate content is determined by calorimetric assay. The role of NO is ascertained by the addition of nitroarginine or (1)-N-methyl arginine, which are specific blockers of NO synthase.

25 **Method B. Vasorelaxation of Phenylephrine-Induced Contracted Rat Artery**

The effects of each of the procyanidins (100 μ g/mL to 0.1 μ g/mL on the rat artery is the target for study of vasorelaxation of phenylephrine-induced
30 contracted rat artery. Isolated rat artery is incubated in the presence or absence of procyanidins (as in Example 14, method D) and alteration of the muscular tone is assessed by visual inspection. Both contraction or relaxation of the ray artery is determined. Then, using
35 other organs, precontraction of the isolated rat artery is induced upon addition of epinephrine. Once the contraction is stabilized, procyanidins are added and contraction or relaxation of the rat artery is determined. The role of NO is ascertained by the
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addition of nitroarginine or (1)-N-methyl arginine. The acetylcholine-induced relaxation of NO, as it is effected by phenylephrine-precontracted rat aorta is shown in Figure 31.

5 Method C. Induction of Hypotension in the Rat

This method is directed to the effect of each procyanidin (as in Example 14, method D) on blood pressure. Rats are instrumented in order to monitor systolic and diastolic blood pressure. Each of the 10 procyanidins are injected intravenously (dosage range = 100 - 0.1 μ g/kg), and alteration of blood pressure is assessed. In addition, the effect of each procyanidin on the alteration of blood pressure evoked by epinephrine is determined. The role of NO is ascertained by the 15 addition of nitroarginine or (1)-N-methyl arginine.

These studies, together with next Example, illustrate that the inventive compounds are useful in modulating vasodilation, and are further useful with respect to modulating blood pressure or addressing 20 coronary conditions, and migraine headache conditions.

Example 27: Effects of Cocoa Polyphenols on Satiety

Using blood glucose levels as an indicator for the signal events which occur *in vivo* for the regulation of appetite and satiety, a series of simple experiments 25 were conducted using a healthy male adult volunteer age 48 to determine whether cocoa polyphenols would modulate glucose levels. Cocoa polyphenols were partially purified from Brazilian cocoa beans according to the methods described by Clapperton et al. (1992). This 30 material contained no caffeine or theobromine. Fasting blood glucose levels were analyzed on a timed basis after ingestion of 10 fl. oz of Dexicola 75 (caffeine free) Glucose tolerance test beverage (Curtin Matheson 091-421) with and without 75mg cocoa polyphenols. This level of 35 polyphenols represented 0.1% of the total glucose of the test beverage and reflected the approximate amount that

would be present in a standard 100g chocolate bar. Blood glucose levels were determined by using the Accu-Chek III blood glucose monitoring system (Boehringer Mannheim Corporation). Blood glucose levels were measured before 5 ingestion of test beverage, and after ingestion of the test beverage at the following timed intervals: 15, 30, 45, 60, 75, 90, 120 and 180 minutes. Before the start of each glucose tolerance test, high and low glucose level controls were determined. Each glucose tolerance test 10 was performed in duplicate. A control test solution containing 75mg cocoa polyphenols dissolved in 10 fl. oz. distilled water (no glucose) was also performed.

Table 11 below lists the dates and control values obtained for each glucose tolerance experiment 15 performed in this study. Figure 32 represents plots of the average values with standard deviations of blood glucose levels obtained throughout a three hour time course. It is readily apparent that there is a substantial increase in blood sugar levels was obtained 20 after ingestion of a test mixture containing cocoa polyphenols. The difference between the two principal glucose tolerance profiles could not be resolved by the profile obtained after ingestion of a solution of cocoa polyphenols alone. The addition of cocoa polyphenols to 25 the glucose test beverage raised the glucose tolerance profile significantly. This elevation in blood glucose levels is within the range considered to be mildly diabetic, even though the typical glucose tolerance profile was considered to be normal (Davidson, I. et al., 30 Eds. Todd - Sandford Clinical Diagnosis by Laboratory Methods 14th edition; W.B. Saunders Co.; Philadelphia, PA 1969 Ch. 10, pp. 550-9). This suggests that the difference in additional glucose was released to the bloodstream, from the glycogen stores, as a result of the 35 inventive compounds. Thus, the inventive compounds can be used to modulate blood glucose levels when in the presence of sugars.

Table 11. Glucose Tolerance Test Dates and Control Results

WEEK	DESCRIPTION	HIGH CONTROL ^a	LOW CONTROL ^b
5	0 Glucose Tolerance	265 mg/dL	53 mg/dL
	1 Glucose Tolerance with 0.1% polyphenols	310	68
	2 Glucose Tolerance	315	66
	4 Glucose Tolerance with 0.1% polyphenols	325	65
	5 0.1% polyphenols	321	66

10

a = Expected range: 253 - 373mg/dL

b = Expected range: 50-80mg/dL

15 The subject also experienced a facial flush (erythema) and lightheadedness following ingestion of the inventive compounds, indicating modulation of vasodilation.

20 The data presented in Tables 12 and 13 illustrates the fact that extracts of the invention pertaining to cocoa raw materials and commercial chocolates, and inventive compounds contained therein can be used as a vehicle for pharmaceutical, veterinary and food science preparations and applications.

Table 13: Procyanidin Levels in Commercial Chocolates
µg/g

Sample	Monomers	Dimers	Trimers	Tetramers	Pentamers	Hexamers	Heptamers and Higher	Total
Brand 1	366	166	113	59	56	23	18	801
Brand 2	344	163	111	45	48	ND*	ND	711
Brand 3	316	181	100	41	40	7	ND	685
Brand 4	310	122	71	27	28	5	ND	563
Brand 5	259	135	90	46	29	ND	ND	559
Brand 6	308	139	91	57	47	14	ND	656
Brand 7	196	98	81	58	54	19	ND	506
Brand 8	716	472	302	170	117	18	ND	1,795
Brand 9	1,185	951	633	298	173	25	21	3,286
Brand 10	1,798	1,081	590	342	307	93	ND	4,211
Brand 11	1,101	746	646	372	347	130	75	3,417
Brand 12	787	335	160	20	10	8	ND	1,320

ND* = None detected.

120

Table 14: Procyanidin Levels in Cocoa Raw Materials
 $\mu\text{g/g}$

Sample	Monomers	Dimers	Trimers	Tetramers	Pentamers	Hexamers	Heptamers and Higher	Total
Unfermented	13,440	6,425	6,401	5,292	4,236	3,203	5,913	44,910
Fermented	2,695	1,538	1,362	740	470	301	277	7,383
Roasted	2,656	1,597	921	337	164	ND*	ND	5,675
Choc. Liquor	2,805	1,446	881	442	184	108	ND	5,866
Cocoa Hulls	114	53	14	ND	ND	ND	ND	181
Cocoa Powder 1% Fat	506	287	112	ND	ND	ND	ND	915
Cocoa Powder 11% Fat	1,523	1,224	680	46	ND	ND	ND	3,473
Red Dutch Cocoa Powder, PH 7.4, 11% fat	1,222	483	103	ND	ND	ND	ND	1,808
Red Dutch Cocoa Powder, PH 8.2, 23% fat	168	144	60	ND	ND	ND	ND	372

ND* = None detected.

Example 28: The Effect of Procyanidins on
Cyclooxygenase 1 & 2

The effect of procyanidins on
5 cyclooxygenase 1 & 2 (COX1/COX2) activities was assessed
by incubating the enzymes, derived from ram seminal
vesicle and sheep placenta, respectively, with
arachidonic acid ($5\mu M$) for 10 minutes at room
temperature, in the presence of varying concentrations of
10 procyanidin solutions containing monomer to decamer and
procyanidin mixture. Turnover was assessed by using PGE2
EIA kits from Interchim (France). Indomethacin was used
as a reference compound. The results are presented in
the following Table, wherein the IC_{50} values are expressed
15 in units of μM (except for S11, which represents a
procyanidin mixture prepared from Example 13, Method A
and where the samples S1 to S10 represent sequentially
procyanidin oligomers (monomer through decamer) as in
Example 14, Method D, and IC_{50} is expressed in units of
20 mg/mL).

SAMPLE #	IC ₅₀ COX-1 (*)	IC ₅₀ COX-2 (*)	RATIO IC ₅₀ COX2/COX1
1	0.074	0.197	2.66
2	0.115	0.444	3.86
3	0.258	0.763	2.96
5	0.154	3.73	24.22
6	0.787	3.16	4.02
7	1.14	1.99	1.75
8	1.89	4.06	2.15
10	2.25	7.2	3.20
9	2.58	2.08	0.81
10	3.65	3.16	0.87
11	0.0487	0.0741	1.52
Indomethacin	0.599	13.5	22.54

15 (*) expressed as uM with the exception of sample
11, which is mg/mL.

20 The results of the inhibition studies are presented in Figures 33 A and B, which shows the effects of Indomethacin on COX1 and COX2 activities. Figures 34 A and B shows the correlation between the degree of polymerization of the procyanidin and IC₅₀ with COX1 and COX2; Figure 35 shows the correlation between IC₅₀ values on COX1 and COX2. And, Figures 36 A through Y show the 25 IC₅₀ values of each sample (S1 - S11) with COX1 and COX2.

These results indicate that the inventive compounds have analgesic, anti-coagulant, and anti-inflammatory utilities. Further, COX2 has been linked to

colon cancer. Inhibition of COX2 activity by the inventive compounds illustrates a plausible mechanism by which the inventive compounds have antineoplastic activity against colon cancer.

5 COX1 and COX2 are also implicated in the synthesis of prostaglandins. Thus, the results in this Example also indicate that the inventive compounds can modulate renal functions, immune responses, fever, pain, mitogenesis, apoptosis, prostaglandin synthesis,
10 ulceration (e.g., gastric), and reproduction. Note that modulation of renal function can affect blood pressure; again implicating the inventive compounds in modulating blood pressure, vasodilation, and coronary conditions (e.g., modulation of angiotensin, bradykinin).

15 Reference is made to Seibert et al., PNAS USA 91:12013-12017 (December, 1994), Mitchell et al., PNAS USA 90:11693-11697 (December 1994), Dewitt et al., Cell 83:345-348 (November 3, 1995), Langenbach et al., Cell 83:483-92 (November 3, 1995) and Sujii et al., Cell 20 83:493-501 (November 3, 1995), Morham et al., Cell 83:473-82 (November 3, 1995).

Reference is further made to Examples 9, 26, and 27. In Example 9, the anti-oxidant activity of inventive compounds is shown. In Example 26, the effect 25 on NO is demonstrated. And, Example 27 provides evidence of a facial vasodilation. From the results in this Example, in combination with Examples 9, 26 and 27, the inventive compounds can modulate free radical mechanisms driving physiological effects. Similarly, lipoxygenase 30 mediated free radical type reactions biochemically directed toward leukotriene synthesis can be modulated by the inventive compounds, thus affecting subsequent physiological effects (e.g., inflammation, immune response, coronary conditions, carcinogenic mechanisms, 35 fever, pain, ulceration).

Thus, in addition to having analgesic properties, there may also be a synergistic effect by the inventive compounds when administered with other analgesics. Likewise, in addition to having 5 antineoplastic properties, there may also be a synergistic effect by the inventive compounds when administered with other antineoplastic agents.

Example 29: Circular Dichroism/Study of Procyanidins

10 CD studies were undertaken in an effort to elucidate the structure of purified procyanidins as in Example 14, Method D. The spectra were collected at 25°C using CD spectrum software AVIV 60DS V4.1f.

15 Samples were scanned from 300nm to 185nm, every 1.00nm, at 1.50nm bandwidth. Representative CD spectra are shown in Figures 43A through G, which show the CD spectra of dimer through octamer.

These results are indicative of the helical nature of the inventive compounds.

20 **Example 30: Inhibitory Effects of Cocoa Procyanidins
on_ *Helicobacter pylori* and *Staphylococcus aureus***

A study was conducted to evaluate the antimicrobial activity of procyanidin oligomers against 25 *Helicobacter pylori* and *Staphylococcus aureus*. Pentamer enriched material was prepared as described in Example 13, Method A and analyzed as described in Example 14, Method C, where 89% was pentamer, and 11% was higher oligomers (n is 6 to 12). Purified pentamer (96.3%) was 30 prepared as described in Example 14, Method D.

Helicobacter pylori and *Staphylococcus aureus* were obtained from the American Type Culture Collection

(ATCC). For *H. pylori*, the vial was rehydrated with 0.5 mL Trypticase Soy broth and the suspension transferred to a slant of fresh TSA containing 5% defibrinated sheep blood. The slant was incubated at 37°C for 3 to 5 days under microaerophilic conditions in anaerobic jars (5 to 10% carbon dioxide; CampyPakPlus, BBL). When good growth was established in the pool of broth at the bottom of the slant, the broth was used to inoculate additional slants of TSA with sheep blood. Because viability decreased with continued subculturing, the broth harvested from the slants was pooled and stored at -80°C. Cultures for assay were used directly from the frozen vials. The *S. aureus* culture was maintained on TSA slants and transferred to fresh slants 24 h prior to use.

A cell suspension of each culture was prepared (*H. pylori*, 10^8 to 10^9 cfu/mL; *S. aureus* 10^6 to 10^7 cfu/mL) and 0.5 mL spread onto TSA plates with 5% sheep blood. Standard assay disks (Difco) were dipped into filter sterilized, serial dilutions of pentamer (23mg/mL into sterile water). The test disks and the blank control disks (sterile water) were placed on the inoculated plates. Control disks containing 80ug metronidazole (inhibitory to *H. pylori*) or 30ug vancomycin (inhibitory to *S. aureus*) (BBL Sensidiscs) were also placed on the appropriate set of plates. The *H. pylori* inoculated plates were incubated under microaerophilic conditions. The *S. aureus* set was incubated aerobically. Zones of inhibition were measured following outgrowth.

**Table 14. Bioassays with pentamer against
Helicobacter pylori and *Staphylococcus aureus***

5	Pentamer Enriched Fraction (mg/ml)	<i>S. aureus</i> Inhibition (mm)	<i>H. pylori</i> Inhibition (mm)
10	0	NI	NI
	15	0	10
	31	10	10
	62	11	11
	125	13	13
	250	15	13
15	Vancomycin standard	15	--
	Metronidazole standard	--	11
20	96% pure pentamer	15	11

NI = no inhibition

Example 31: NO DEPENDENT HYPOTENSION IN THE GUINEA PIG

The effect of five cocoa procyanidin fractions on guinea pig blood pressure were investigated. Briefly, 25 guinea pigs (approximately 400g body weight; male and female) were anesthetized upon injection of 40 mg/kg sodium pentobarbital. The carotid artery was cannulated for monitoring of the arterial blood pressure. Each of the five cocoa procyanidin fractions was injected

intravenously (dose range 0.1 mg/kg - 100 mg/kg) through the jugular vein. Alterations of blood pressure were recorded on a polygraph. In these experiments, the role of NO was ascertained by the administration of L-N-5 methylarginine (1 mg/kg) ten minutes prior to the administration of cocoa procyanidin fractions.

Cocoa procyanidin fractions were prepared and analyzed according to the procedures described in U.S. Patent 5,554,645, hereby incorporated herein by 10 reference.

Fraction A: Represents a preparative HPLC fraction comprised of monomers-tetramers. HPLC analysis revealed the following composition:

Monomers 47.2%

15 Dimers 23.7

Trimers 18.7

Tetramers 10.3

Fraction B: Represents a preparative HPLC fraction 20 comprised of pentamers-decamers. HPLC analysis revealed the following composition:

Pentamers 64.3%

Hexamers 21.4

25 Heptamers 7.4

Octamers 1.9

Nonamers 0.9

Decamers 0.2

Fraction C: Represents an enriched cocoa procyanidin fraction used in the preparation of Fractions A and B (above). HPLC analysis revealed the following composition:

5	Monomers	34.3%
	Dimers	17.6
	Trimers	16.2
	Tetramers	12.6
	Pentamers	8.5
10	Hexamers	5.2
	Heptamers	3.1
	Octamers	1.4
	Nonamers	0.7
	Decamers	0.3

15

Fraction D: Represents a procyanidin extract prepared from a milk chocolate. HPLC analysis revealed a composition similar to that listed in the Table 12 for Brand 8. Additionally, caffeine 10% and theobromine 6.3% were present.

20
25 Fraction E: Represents a procyanidin extract prepared from a dark chocolate prepared with alkalized liquor. HPLC analysis revealed a composition similar to that listed in the Table 12 for Brand 12. Additionally, caffeine 16.0% and theobromine 5.8% were present.

In three separate experiments, the effects of administering 10mg/kg cocoa procyanidin fractions on arterial blood pressure of anesthetized guinea pigs was 5 investigated. Upon intravenous injection, procyanidin fractions A and E evoked a decrease in blood pressure of about 20%. This decrease was only marginally different from that obtained from a solvent (DMSO) control (15 ± 5%, n=5). In contrast, procyanidin fractions B, C and D 10 (10mg/kg) induced marked decreases in blood pressure, up to 50-60% for C. In these experiments the order of hypotensive effect was as follows: C>B>D>>A=E.

Typical recordings of blood pressure elicited after injection of procyanidin fractions appear in Figure 15 50A for fraction A and Figure 50B for fraction C. Figure 51 illustrates the comparative effects on blood pressure by these fractions.

The possible contribution of NO in the 20 hypotension in the guinea pig induced by administration of fraction C was analyzed using L-N-methyl arginine (LNMMa). This pharmacological agent inhibits the formation of NO by inhibiting NO synthase. LNMMa was administered at the dose of 1 mg/kg, ten minutes prior to injection of the cocoa procyanidin fractions. As shown 25 in Figure 52, treatment of the animals with LNMMa completely blocked the hypotension evoked by the procyanidin fraction C. Indeed, following treatment with this inhibitor, the alterations of blood pressure produced by fraction C were similar to those noted with 30 solvent alone.

Example 32: Effect of Cocoa Procyanidin Fractions on NO

**Production in Human Umbilical Vein
Endothelial Cells**

130

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and cultures were carried out according to the manufacturer's specifications. HUVEC cells were seeded at 5,000 cells/cm² in 12-well plates (Falcon). After the third passage under the same conditions, they were allowed to reach confluence. The supernatant was renewed with fresh medium containing defined concentrations of bradykinin (25,50 and 100nM) or cocoa procyanidin fractions A-E (100µg/mL) as described in example 31. The culture was continued for 24 hr. and the cell free supernatants were collected and stored frozen prior to assessment of NO content as described below. In selected experiments, the NO synthase (NOS) antagonist, Nω-nitro-L-arginine methyl ester (L-NAME, 10µM) was added to assess the involvement of NOS in the observed NO production.

HUVEC NO production was estimated by measuring nitrite concentration in the culture supernatant by the Griess reaction. Griess reagent was 1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride. Briefly, 50µL aliquots were removed from the various supernatants in quadruplicate and incubated with 150µL of the Griess reagent. The absorbency at 540 nm was determined in a multiscan (Labsystems Multiskans MCC/340) apparatus. Sodium nitrite was used at defined concentrations to establish standard curves. The absorbency of the medium without cells (blank) was subtracted from the value obtained with the cell containing supernatants.

Figure 53 illustrates the effect of bradykinin on NO production by HUVEC where a dose dependent release of NO was observed. The inhibitor L-NAME completely inhibited the bradykinin induced NO release.

Figure 54 illustrates the effect of the cocoa procyanidin fractions on NO production by HUVEC cells.

Fractions B, C and D induced a moderate but significant amount of NO production by HUVEC. By far, Fraction C was the most efficient fraction to induce NO formation as assessed by the production of nitrites, while Fraction E 5 was nearly ineffective. The effect of Fraction C on NO production was dramatically reduced in the presence of L-NAME. Interestingly, Fractions B, C and D contained higher amounts of procyanidin oligomers than Fractions A and E. A distinguishing difference between Fractions D 10 and E was that E was prepared from a dark chocolate which used alkalized cocoa liquor as part of the chocolate recipe. Alkalization leads to a base catalyzed polymerization of procyanidins which rapidly depletes the levels of these compounds. An analytical comparison of 15 procyanidin levels found in these types of chocolate appear in the Table 12, where Brand 12 is a dark chocolate prepared with alkalized cocoa liquor and Brand 11 is a typical milk chocolate. Thus, extracts obtained from milk chocolates contain high proportions of 20 procyanidin oligomers which are capable of inducing NO. The addition of the NO inhibitor L-NMMA to the Fraction C sample clearly led to the inhibition of NO. The results obtained from the procyanidin fractions were consistent to those observed with the bradykinin induced NO 25 experiment (see Figure 53).

As in the case of the HUVEC results, cocoa procyanidin fraction C elicited a major hypotensive effect in guinea pigs, whereas fractions A and E were the least effective. Again, the presence of high molecular 30 weight procyanidin oligomers were implicated in the modulation of NO production.

**Example 33: Effect of Cocoa Procyanidin Fractions on
Macrophage NO Production**

Fresh, human heparinized blood (70 mL) was 35 added with an equal volume of phosphate buffer saline

(PBS) at room temperature. A Ficoll-Hypaque solution was layered underneath the blood-PBS mixture using a 3mL Ficoll-Hypaque to 10mL blood-PBS dilution ratio. The tubes were centrifuged for 30 minutes at 2,000 rpm at 18-
5 20°C. The upper layer containing plasma and platelets was discarded. The mononuclear cell layer was transferred to another centrifuge tube and the cells were washed 2X in Hanks balanced saline solution. The mononuclear cells were resuspended in complete RPMI 1640
10 supplemented with 10% fetal calf serum, counted and the viability determined by the trypan blue exclusion method. The cell pellet was resuspended in complete RPMI 1640 supplemented with 20% fetal calf serum to a final concentration of 1×10^6 cells/mL. Aliquots of the cell
15 suspension were plated into a 96 well culture plate and rinsed 3X with RPMI 1640 supplemented with 10% fetal calf serum and the nonadherent cells (lymphocytes) were discarded.

These cells were incubated for 48 hours in the
20 presence or absence of five procyanidin fractions described in Example 31. At the end of the incubation period, the culture media were collected, centrifuged and cell free supernatants were stored frozen for nitrate assay determinations.

25 Macrophage NO production was determined by measuring nitrite concentrations by the Greiss reaction. Greiss reagent was 1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride. Briefly, 50µL aliquots were removed from the supernatants in quadruplicate and
30 incubated with 150µL of the Greiss reagent. The absorbency at 540 nm was determined in a multiscan (Labsystems Multiskans MCC/340) apparatus. Sodium nitrite was used at defined concentrations to establish standard curves. The absorbency of the medium without
35 cells (blank) was subtracted from the value obtained with the cell containing supernatants.

In a separate experiment, macrophages were primed for 12 hours in the presence of 5U/mL gamma-interferon and then stimulated with 10 μ g/mL LPS for the next 36 hours in the presence or absence of 100 μ g/mL of 5 the five procyanidin fractions.

Figure 55 indicates that only procyanidin fraction C, at 100 μ g/mL, could induce NO production by monocytes/macrophages. Basal NO production by these cells was undetectable and no nitrite could be detected 10 in any of the cocoa procyanidin fractions used at 100 μ g/mL. Figure 56 indicates that procyanidin fractions A and D enhanced LPS-induced NO production by γ -interferon primed monocytes/macrophages. Procyanidin fraction C was marginally effective, since LPS-stimulated 15 monocytes/macrophages cultured in the absence of procyanidin fractions produced only 4 μ mole/10⁵ cells/48 hours. γ -Interferon alone was ineffective in inducing NO.

Collectively, these results demonstrate that 20 mixtures of the inventive compounds used at specific concentrations are capable of inducing monocyte/macrophage NO production both independent and dependent of stimulation by LPS or cytokines.

From the foregoing, it is clear that the 25 extract and cocoa polyphenols, particularly the inventive compounds, as well as the compositions, methods, and kits, of the invention have significant and numerous utilities.

The antineoplastic utility is clearly 30 demonstrated by the *in vivo* and *in vitro* data herein and shows that inventive compounds can be used instead of or in conjunction with conventional antineoplastic agents.

The inventive compounds have antioxidant activity like that of BHT and BHA, as well as oxidative

stability. Thus, the invention can be employed in place of or in conjunction with BHT or BHA in known utilities of BHA and BHT, such as an antioxidant, for instance, an antioxidant; food additive.

5 The invention can also be employed in place of or in conjunction with topoisomerase II-inhibitors in the presently known utilities therefor.

10 The inventive compounds can be used in food preservation or preparation, as well as in preventing or treating maladies of bacterial origin. Simply the inventive compounds can be used as an antimicrobial.

15 The inventive compounds can also be used as a cyclo-oxygenase and/or lipoxygenase, NO or NO-synthase, or blood or in vivo glucose modulator, and are thus useful for treatment or prevention or modulation of pain, fever, inflammation coronary conditions, ulceration, carcinogenic mechanisms, vasodilation, as well as an analgesic, anti-coagulant anti-inflammatory and an immune response modulator.

20 Further, the invention comprehends the use of the compounds or extracts as a vehicle for pharmaceutical preparations. Accordingly, there are many compositions and methods envisioned by the invention. For instance, antioxidant or preservative compositions, topoisomerase 25 II-inhibiting compositions, methods for preserving food or any desired item such as from oxidation, and methods for inhibiting topoisomerase II. The compositions can comprise the inventive compounds. The methods can comprise contacting the food, item or topoisomerase II with the respective composition or with the inventive 30 compounds. Other compositions, methods and embodiments of the invention are apparent from the foregoing.

In this regard, it is mentioned that the invention is from an edible source and, that the activity

in vitro can demonstrate at least some activity *in vivo*; and from the *in vitro* and *in vivo* data herein, doses, routes of administration, and formulations can be obtained without undue experimentation

5 **Example 34. Micellar Electrokinetic Capillary**
Chromatography of Cocoa Procyanidins

A rapid method was developed using micellar electrokinetic capillary chromatography (MECC) to separate procyanidin oligomers. The method is a
10 modification of that reported by Delgado et al., 1994. The MECC method requires only 12 minutes to achieve the same separation as that obtained by a 70 minute normal phase HPLC analysis. Figure 57 represents a MECC separation of cocoa procyanidins obtained by Example 2.

15 MECC Conditions:

The cocoa procyanidin extract was prepared by the method described in Example 2 and dissolved at a concentration of 1 mg/mL in MECC buffer consisting of 200mM boric acid, 50mM sodium dodecyl sulfate
20 (electrophoresis pure) and NaOH to adjust to pH = 8.5.

The sample was passed through a 0.45um filter and electrophoresed using a Hewlett Packard HP-3D CZE System operated at the following conditions:

Inlet buffer: Run buffer as described above
25 Outlet buffer: Run buffer as described above

Capillary: 50cm x 75um i.d. uncoated fused
silica

Detection: 200nm, with Diode Array Detector

Injection: 50 mBar for 3 seconds (150 mBar
30 sec)

Voltage: 6 watts

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Amperage: System limit (<300uA)

Temperature: 25°C

Capillary Condition: 5 min flush with run
5 buffer before and after each run.

This method can be modified by profiling temperature, pressure, and voltage parameters, as well as including organic modifiers and chiral selective agents in the run buffer.

10 Example 35. MALDI - TOF/MS Analysis of Procyanidin Oligomers with Metal Salt Solutions

A series of MALDI -TOF/MS analyses were performed on trimers combined with various metal salt 15 solutions to determine whether cation adducts of the oligomer could be detected. The significance of the experiment was to provide evidence that the procyanidin oligomers play a physiological role *in vitro* and *in vivo* by sequestering or delivering metal cations important to 20 physiological processes and disease.

The method used was as described in Example 15. Briefly, 2uL of 10mM solutions of zinc sulfate dihydrate, calcium chloride, magnesium sulfate, ferric chloride hexahydrate, ferrous sulfate heptahydrate, and cupric sulfate were individually combined with 4uL of a trimer 25 (10mg/mL) purified to apparent homogeneity as described in Example 14, and 44uL of DHB added.

The results (Figures 58A-F) showed [Metal-Trimer + H]⁺ ions for copper and iron (ferrous and 30 ferric) whose m/z values matched \pm 1 amu standard deviation value for the theoretical calculated masses. The [Metal-Trimer + H]⁺ masses for calcium and magnesium

could not be unequivocally resolved from the [Metal-Trimer + H]⁺ masses for sodium and potassium, whose m/z values were within the ± 1 amu standard deviation values. No [Zn⁺² - Trimer + H]⁺ ion could be detected. Since some 5 of these cations are multi-valent, the possibility for multimetal-oligomer(s) ligand species and /or metal-metoligomer species were possible. However, scanning for these adducts at their predicted masses proved unsuccessful.

10 The results shown above for copper, iron, calcium, magnesium and zinc may be used as general teachings for subsequent analysis of the reaction between other metal ions and the inventive compounds, taking into account such factors as oxidation state and the relative 15 position in the periodic table of the ion in question.

Example 36. MALDI - TOF/MS Analysis of High Molecular

Weight Procyanidin Oligomers

An analytical examination was made on GPC
20 eluants associated with high molecular weight procyanidin oligomers as prepared in Example 3, Method A. The objective was to determine whether procyanidin oligomers with n > 12 were present. If present, these oligomers represent additional compounds of the invention.
25 Adjustments to existing methods of isolation, separation and purification embodied in the invention can be made to obtain these oligomers for subsequent *in vitro* and *in vivo* evaluation for anti-cancer, anti-tumor or antineoplastic activity, antioxidant activity, inhibit
30 DNA topoisomerase II enzyme, inhibit oxidative damage to DNA, and have antimicrobial, NO or NO-synthase, apoptosis, platelet aggregation, and blood or *in vivo* glucose modulating activities, as well as efficacy as non-steroidal antiinflammatory agents.

Figure 59 represents a MALDI-TOF mass spectrum of the GPC eluant sample described above. The $[M + Na]^+$ and/or $[M + K]^+$ and/or $[M + 2Na]^+$ ions characterizing procyanidin oligomers representative of tetramers through 5 octadecamers are clearly evident.

It was learned that an acid and heat treatment will cause the hydrolysis of procyanidin oligomers. Therefore, the invention comprehends the controlled hydrolysis of high molecular weight procyanidin oligomers 10 (e.g. where n is 13 to 18) as a method to prepare lower molecular weight procyanidin oligomers (e.g. where n is 2 to 12).

Example 37. Dose Response Relationships of Procyanidin Oligomers and Canine and Feline Cell

15 Lines

The dose response effects of procyanidin oligomers were evaluated against several canine and feline cell lines obtained from the Waltham Center for 20 Pet Nutrition, Waltham on-the-Wolds, Melton Mowbray, Leicestershire, U.K. These cell lines were

Canine normal kidney GH cell line;

Canine normal kidney MDCK cell line;

Feline normal kidney CRFK cell line; and

25 Feline lymphoblastoid FeA cell line producing leukemia virus which were cultured under the conditions described in Example 8, Method A.

Monomers and procyanidin oligomers, where n is 2 to 10 were purified as described in Example 14, Method 30 D. The oligomers were also examined by analytical normal phase HPLC as described in Example 14, Method C, where the following results were obtained.

	<u>Procyanidin</u>	<u>% Purity by HPLC</u>
	Monomers	95.4
	Dimers	98.0
	Trimers	92.6
5	Tetramers	92.6
	Pentamers	93.2
	Hexamers	89.2 (Contains 4.4% pentamers)
10	Heptamers	78.8 (Contains 18.0% hexamers)
	Octamers	76.3 (contains 16.4% heptamers)
	Nonamers	60.3 (Contains 27.6% octamers)
15	Decamers	39.8 (Contains 22.2% nonamers, 16.5% octamers, and 13.6% heptamers)

20 In those cases where the purity of the oligomer is < 90%, methods embodied in the invention are used for their repurification.

25 Each cell line was dosed with monomers and each procyanidin oligomer at 10ug/mL, 50ug/mL and 100ug/mL and the results shown in Figures 60 - 63. As shown in the Figures, high dose (100ug/mL) administration of individual oligomers produced similar inhibitory effects on the feline FeA lymphoblastoid and feline normal kidney CRFK cell lines. In these cases, cytotoxicity appeared

with the tetramer, and increasingly higher oligomers elicited increasingly higher cytotoxic effects. By contrast, high dose (100ug/mL) administration of individual oligomers to canine GH and MDCK normal kidney 5 cell lines required a higher oligomer to initiate the appearance of cytotoxicity. For the canine GH normal kidney cell line, cytotoxicity appeared with the pentamer. For the canine MDCK normal kidney cell line, cytotoxicity appeared with the hexamer. In both of these 10 cases, the administration of higher oligomers produced increasing levels of cytotoxicity.

Example 38. Tablet Formulations

A tablet formulation was prepared using cocoa solids obtained by methods described in U.S. Application 15 Serial No. 08/709,406 filed 6 September 1996, hereby incorporated herein by reference. Briefly, this edible material is prepared by a process which enhances the natural occurrence of the compounds of the invention in contrast to their levels found in traditionally processed 20 cocoa, such that the ratio of the initial amount of the compounds of the invention found in the unprocessed bean to that obtained after processing is less than or equal to 2. For simplicity, this cocoa solids material is designated herein as CP-cocoa solids. The inventive 25 compound or compounds, e.g., in isolated and/or purified form may be used in tablets as described in this Example, instead of or in combination with CP-cocoa solids.

A tablet formula comprises the following (percentages expressed as weight percent):

30	CP-cocoa solids	24.0%
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4-Fold Natural vanilla extract	
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(Bush Boake Allen)	1.5%
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	Magnesium stearate (dry lubricant) (AerChem, Inc.)	0.5%
	Dipac tabletting sugar 5 (Amstar Sugar Corp.)	37.0%
	Xylitol (American Xyrofin, Inc.)	37.0%

		100.0%

10 The CP-cocoa solids and vanilla extract are
blended together in a food processor for 2 minutes. The
sugars and magnesium stearate are gently mixed together,
followed by blending in the CP-cocoa solids/vanilla mix.
This material is run through a Manesty Tablet Press (B3B)
15 at maximum pressure and compaction to produce round
tablets (15mm x 5mm) weighing 1.5 - 1.8 gram. Another
tablet of the above mentioned formula was prepared with a
commercially available low fat natural cocoa powder (11%
fat) instead of the CP-cocoa solids (11% fat). Both
20 tablet formulas produced products having acceptable
flavor characteristics and texture attributes.

25 An analysis of the two tablet formulas was
performed using the procedures described in Example 4,
Method 2. In this case, the analysis focused on the
concentration of the pentamer and the total level of
monomers and compounds of the invention where n is 2 to
12 which are reported below.

	Tablet sample	pentamer (ug/g)	total (ug/g)	pentamer (ug/1.8g serving)	total (ug/1.8g serving)
5	tablet with CP-cocoa solids	239	8,277	430	14,989
10	tablet with commercial low fat cocoa powder	ND	868	ND	1563

ND = not detected

15 The data clearly showed a higher level of pentamer and total level of compounds of the invention in the CP-cocoa solids tablet than in the other tablet formula. Thus, tablet formulas prepared with CP-cocoa solids are an ideal delivery vehicle for the oral
20 administration of compounds of the invention, for pharmaceutical, supplement and food applications.

 The skilled artisan in this area can readily prepare other tablet formulas covering a wide range of flavors, colors, excipients, vitamins, minerals, OTC
25 medicaments, sugar fillers, UV protectants (e.g., titanium dioxide, colorants, etc.), binders, hydrogels, and the like except for polyvinyl pyrrolidone which would irreversibly bind the compounds of the invention or combination of compounds. The amount of sugar fillers may
30 be adjusted to manipulate the dosages of the compounds of the invention or combination of compounds.

Many apparent variations of the above are self-evident and possible without departing from the spirit and scope of the example.

Example 39. Capsule Formulations

5 A variation of Example 38 for the oral delivery of the compounds of the invention is made with push-fit capsules made of gelatin, as well as soft sealed capsules made of gelatin and a plasticizer such as glycerol. The push-fit capsules contain the compound of the invention
10 or combination of compounds or CP-cocoa solids as described in Examples 38 and 40 in the form of a powder which can be optionally mixed with fillers such as lactose or sucrose to manipulate the dosages of the compounds of the invention. In soft capsules, the
15 compound of the invention or combination of compounds or CP-cocoa solids are suspended in a suitable liquid such as fatty oils or cocoa butter or combinations therein. Since an inventive compound or compounds may be light-sensitive, e.g., sensitive to UV, a capsule can contain
20 UV protectants such as titanium dioxide or suitable colors to protect against UV. The capsules can also contain fillers such as those mentioned in the previous Example.

Many apparent variations of the above are self-evident and possible to one skilled in the art without departing from the spirit and scope of the example.

Example 40. Standard of Identity (SOI) and Non-Standard of Identity (non-SOI) Dark and Milk Chocolate Formulations

Formulations of the compounds of the invention or combination of compounds derived by methods embodied in the invention can be prepared into SOI and non-SOI

dark and milk chocolates as a delivery vehicle for human and veterinary applications. Reference is made to copending U.S. Application Serial No. 08/709,406, filed September 6, 1996, hereby incorporated herein by reference. USSN 08/709,406 relates to a method of producing cocoa butter and/or cocoa solids having conserved levels of the compounds of the invention from cocoa beans using a unique combination of processing steps. Briefly, the edible cocoa solids obtained by this process conserves the natural occurrence of the compounds of the invention in contrast to their levels found in traditionally processed cocoa, such that the ratio of the initial amount of the compounds of the invention found in the unprocessed bean to that obtained after processing is less than or equal to 2. For simplicity, this cocoa solids material is designated herein as CP-cocoa solids. The CP-cocoa solids are used as a powder or liquor to prepare SOI and non-SOI chocolates, beverages, snacks, baked goods, and as an ingredient for culinary applications.

The term "SOI chocolate" as used herein shall mean any chocolate used in food in the United States that is subject to a Standard of Identity established by the U.S. Food and Drug Administration under the Federal Food, Drug and Cosmetic Act. The U.S. definitions and standards for various types of chocolate are well established. The term "non-SOI chocolate" as used herein shall mean any nonstandardized chocolates which have compositions which fall outside the specified ranges of the standardized chocolates.

Examples of nonstandardized chocolates result when the cocoa butter or milk fat are replaced partially or completely; or when the nutritive carbohydrate sweetener is replaced partially or completely; or flavors imitating milk, butter, cocoa powder, or chocolate are added or other additions or deletions in the formula are

made outside the U.S. FDA Standards of Identity for chocolate or combinations thereof.

As a confection, chocolate can take the form of solid pieces of chocolate, such as bars or novelty shapes, and can also be incorporated as a component of other, more complex confections where chocolate is optionally combined with any Flavor & Extract Manufacturers Association (FEMA) material, natural juices, spices, herbs and extracts categorized as natural-flavoring substances; nature-identical substances; and artificial flavoring substances as defined by FEMA GRAS lists, FEMA and FDA lists, Council of Europe (CoE) lists, International Organization of the Flavor Industry (IOFI) adopted by the FAO/WHO Food Standard Programme, Codex Alimentarius, and Food Chemicals Codex and generally coats other foods such as caramel, nougat, fruit pieces, nuts, wafers or the like. These foods are characterized as microbiologically shelf-stable at 65-85°F under normal atmospheric conditions.

Other complex confections result from surrounding with chocolate soft inclusions such as cordial cherries or peanut butter. Other complex confections result from coating ice cream or other frozen or refrigerated desserts with chocolate. Generally, chocolate used to coat or surround foods must be more fluid than chocolates used for plain chocolate solid bars or novelty shapes.

Additionally, chocolate can also be a low fat chocolate comprising a fat and nonfat solids, having nutritive carbohydrate sweetener(s), and an edible emulsifier. As to low fat chocolate, reference is made to U.S. Patent Nos. 4,810,516, 4,701,337, 5,464,649, 5,474,795, and WO 96/19923.

Dark chocolates derive their dark color from the amount of chocolate liquor, or alkalized liquor or cocoa solids or alkalized cocoa solids used in any given

formulation. However, the use of alkalized cocoa solids or liquor would not be used in the dark chocolate formulations in the invention, since Example 27, Table 13 teaches the loss of the compounds of the invention due to 5 the alkalization process.

Examples of formulations of SOI and non-SOI dark and milk chocolates are listed in Tables 16 and 17. In these formulations, the amount of the compounds of the invention present in CP-cocoa solids was compared to the 10 compounds of the invention present in commercially available cocoa solids.

The following describes the processing steps used in preparing these chocolate formulations.

Process for non-SOI Dark Chocolate

- 15 1. Keep all mixers and refiners covered throughout process to avoid light.
2. Batch all the ingredients excluding 40% of the free fat (cocoa butter and anhy. milk fat) maintaining temperature between 30-35°C.
- 20 3. Refine to 20 microns.
4. Dry conche for 1 hour at 35°C.
5. Add full lechithin and 10% cocoa butter at the beginning of the wet conche cycle; wet conche for 1 hour.
- 25 6. Add all remaining fat, standardize if necessary and mix for 1 hour at 35°C.
7. Temper, mould and package chocolate.

Process for SOI Dark Chocolate

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1. Batch all ingredients excluding milk fat at a temperature of 60°C.
2. Refine to 20 microns.
3. Dry conche for 3.5 hours at 60°C.
- 5 4. Add lecithin and milk fat and wet conche for 1 hour at 60°C.
5. Standardize if necessary and mix for 1 hour at 35°C.

Temper, mould and package chocolate.

10 Process for non-SOI Milk Chocolate

1. Keep all mixers and refiners covered throughout process to avoid light.
2. Batch sugar, whole milk powder, malted milk powder, 15 and 66% of the cocoa butter, conche for 2 hours at 75°C.
3. Cool batch to 35°C and add cocoa powder, ethyl vanillin, chocolate liquor and 21% of cocoa butter, mix 20 minutes at 35°C.
4. Refine to 20 microns.
- 20 5. Add remainder of cocoa butter, dry conche for 1.5 hour at 35°C.
6. Add anhy. milk fat and lecithin, wet conche for 1 hour at 35°C.
7. Standardize, temper, mould and package the chocolate.

25

Process for SOI Milk Chocolate

1. Batch all ingredients excluding 65% of cocoa butter and milk fat at a temperature of 60°C.
2. Refine to 20 microns.
3. Dry conche for 3.5 hours at 60°C.
- 5 4. Add lecithin, 10% of cocoa butter and anhy. milk fat; wet conche for 1 hour at 60°C.
5. Add remaining cocoa butter, standardize if necessary and mix for 1 hour at 35°C.
6. Temper, mould and package the chocolate.

10

The CP-cocoa solids and commercial chocolate liquors used in the formulations were analyzed for the pentamer and total level of monomers and compounds of the invention where n is 2 to 12 as described in Method 2, 15 Example 4 prior to incorporation in the formulations. These values were then used to calculate the expected levels in each chocolate formula as shown in Tables 16 and 17. In the cases for the non-SOI dark chocolate and non-SOI milk chocolate, their products were similarly 20 analyzed for the pentamer, and the total level of monomers and the compounds of the invention where n is 2 to 12. The results appear in Tables 16 and 17.

The results from these formulation examples indicated that SOI and non-SOI dark and milk chocolates 25 formulated with CP-cocoa solids contained approximately 6.5 times more expected pentamer, and 3.5 times more expected total levels in the SOI and non-SOI dark chocolates; and approximately 4.5; 7.0 times more expected pentamer and 2.5; 3.5 times more expected total 30 levels in the SOI and non-SOI milk chocolates, respectively.

Analyses of some of the chocolate products were not performed since the difference between the expected levels of the compounds of the invention present in finished chocolates prepared with CP-cocoa solids were dramatically higher than those formulas prepared with commercially available cocoa solids. However, the effects of processing was evaluated in the non-SOI dark and milk chocolate products. As shown in the tables, a 25-50% loss of the pentamer occurred, while slight differences in total levels were observed. Without wishing to be bound by any theory, it is believed that these losses are due to heat and/or low chain fatty acids from the milk ingredient (e.g. acetic acid, propionic acid and butyric acid) which can hydrolyze the oligomers (e.g. a trimer can hydrolyze to a monomer and dimer). Alternatively, time consuming processing steps can allow for oxidation or irreversible binding of the compounds of the invention to protein sources within the formula. Thus, the invention comprehends altering methods of chocolate formulation and processing to address these effects to prevent or minimize these losses.

The skilled artisan will recognize many variations in these examples to cover a wide range of formulas, ingredients, processing, and mixtures to rationally adjust the naturally occurring levels of the compounds of the invention for a variety of chocolate applications.

Table 16. Dark Chocolate Formulas Prepared with non-Alkalized Cocoa Ingredients

		Non-SOI Dark Chocolate Using CP-cocoa solids	SOI Dark Chocolate Using CP-Cocoa Solids	SOI Dark Chocolate Using Commercial Cocoa Solids
	Formulation:	Formulation:	Formulation:	Formulation:
5	41.49% Sugar 3% whole milk powder 26% CP-cocoa solids 4.5% com. liquor 21.75% cocoa butter 2.75% anhy. milk fat 0.01% vanillin 0.5% lecithin	41.49% sugar 3% whole milk powder 52.65% CP-liquor 2.35% anhy. milk fat 0.01% vanillin 0.5% lecithin	41.49% sugar 3% whole milk powder 52.65% com. liquor 2.35% anhy. milk fat 0.01% vanillin 0.5% lecithin	41.49% sugar 3% whole milk powder 52.65% com. liquor 2.35% anhy. milk fat 0.01% vanillin 0.5% lecithin
10	Total fat: 31% Particle size: 20 microns	Total fat: 31% Particle size: 20 microns	Total fat: 31% Particle size: 20 microns	Total fat: 31% Particle size: 20 microns
15				
				Expected Levels of pentamer and total oligomeric procyanidins (monomers and n = 2-12; units of µg/g)
20	Pentamer: 1205 Total: 13748	Pentamer: 1300 Total: 14646	Pentamer: 185 Total: 3948	Actual Levels of pentamer and total oligomeric procyanidins (monomers and n = 2-12; units of µg/g)
	Pentamer: 561 Total: 14097	Not performed	Not performed	

Table 17. Milk Chocolate Formulas Prepared with non-Alkalized Cocoa Ingredients

	Non-SOI Milk Chocolate Using CP-cocoa solids	SOI Milk Chocolate Using CP-Cocoa Solids	SOI Milk Chocolate Using Commercial Cacao Solids
Formulation:	Formulation:	Formulation:	Formulation:
5	46.9965 % Sugar 15.5% whole milk powder 4.5% CP-cocoa solids 5.5% com. liquor 21.4% cocoa butter 1.6% anhy. milk fat 0.035% vanillin 0.5% lecithin 4.0% malted milk powder Total fat: 31.75%	46.9965% sugar 15.5% whole milk powder 13.9% CP-Liquor 1.6% anhy. milk fat 0.0035% vanillin 0.5% lecithin 17.5% cocoa butter 4.0% malted milk powder Total fat: 31.75%	46.9965% sugar 15.5% whole milk powder 13.9% com. liquor 1.60% anhy. milk fat 0.0035% vanillin 0.5% lecithin 17.5% cocoa butter 4.0% malted milk powder Total fat: 31.75%
10	0.35% vanillin 0.5% lecithin 4.0% malted milk powder Particle size: 20 microns	0.35% vanillin 0.5% lecithin 4.0% malted milk powder Particle size: 20 microns	0.35% vanillin 0.5% lecithin 4.0% malted milk powder Particle size: 20 microns
15			

Expected Levels of pentamer and total oligomeric procyandins (monomers and n = 2-12; units of $\mu\text{g/g}$)

Pentamer: 225	Pentamer: 343	Pentamer: 49
Total: 2734	Total: 3867	Total: 1042

Actual Levels of pentamer and total oligomeric procyandins (monomers and n = 2-12; units of $\mu\text{g/g}$)

Pentamer: 163	Not performed	Not performed
Total: 2399		

Example 41. Hydrolysis of Procyanidin Oligomers

Example 14, Method D describes the preparation normal phase HPLC procedure to purify the compounds of the invention. The oligomers are obtained as fractions dissolved in mobile phase. Solvent is then removed by standard vacuum distillation (20-29 in. Hg: 40°C) on a Rotovap apparatus. It was observed that losses of a particular oligomer occurred with increases in smaller oligomers when the vacuum distillation residence time was prolonged or temperatures >40°C were used.

The losses of a particular oligomer with accompanying increases in smaller oligomers was attributed to a time-temperature acid hydrolysis from residual acetic acid present in the mobile phase solvent mixture. This observation was confirmed by the following experiment where 100mg of hexamer was dissolved in 50mL of the mobile phase containing methylene chloride, acetic acid, water, and methanol (see Example 14, Method D for solvent proportions) and subjected to a time-temperature dependent distillation. At specific times, an aliquot was removed for analytical normal phase HPLC analysis as described in Example 4, Method 2. The results are illustrated in Figures 64 and 65, where hexamer levels decreased in a time-temperature dependent fashion. Figure 65 illustrates the appearance of one of the hydrolysis products (Trimer) in a time-temperature dependent fashion. Monomer and other oligomers (dimer to pentamer) also appeared in a time-temperature dependent fashion.

These results indicated that extreme care and caution must be taken during the handling of the inventive polymeric compounds.

The results provided above, together with that found in Examples 5, 15, 18, 19, 20 and 29, demonstrate that the method described above can be used to complement

other methods embodied in the invention to identify any given oligomer of the invention.

For instance, the complete hydrolysis of any given oligomer which yields exclusively (+)-catechin or 5 (-)-epicatechin eliminates many "mixed" monomer-based oligomer structure possibilities and reduces the stereochemical linkage possibilities characteristic for each monomer comprising any given oligomer.

Further, the complete hydrolysis of any given 10 oligomer which yields both (+)-catechin and (-)- epicatechin in specific proportions provides the skilled artisan with information on the monomer composition of any given oligomer, and hence, the stereochemical linkage possibilities characteristic for each monomer comprising 15 the oligomer.

The skilled artisan would recognize the fact that acid catalyzed epimerization of individual monomers can occur and suitable control experiments and nonvigorous hydrolysis conditions should be taken into 20 account (e.g., the use of an organic acids, such as acetic acid, in lieu of concentrated HCl, HNO₃, etc).

Having thus described in detail the preferred embodiments of the present invention, it is to be understood that the invention defined by the appended 25 claims is not to be limited by particular details set forth in the above descriptions as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

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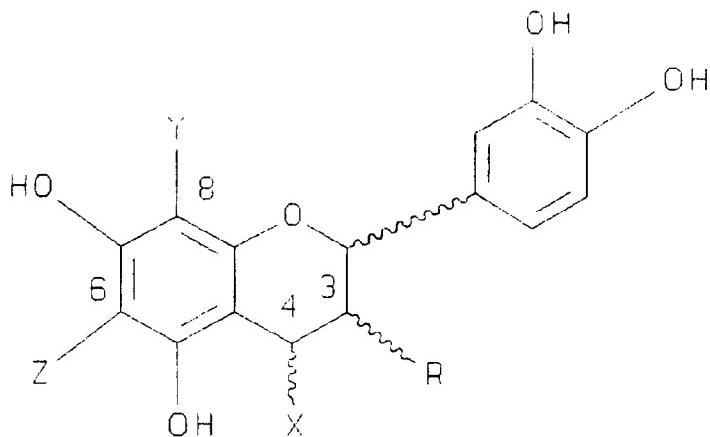
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WHAT IS CLAIMED IS:

1. A polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 3 to 18, such that there is at least one terminal monomeric unit A, and a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond for an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety, and

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof.

2. The compound of claim 1 wherein n is 5.

3. The compound of claim 1 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

4. The compound of claim 1 which is isolated from a natural source.

5. The compound of claim 4 wherein the natural source is a *Theobroma* or *Herrania* species or inter- or intra-species specific crosses thereof.

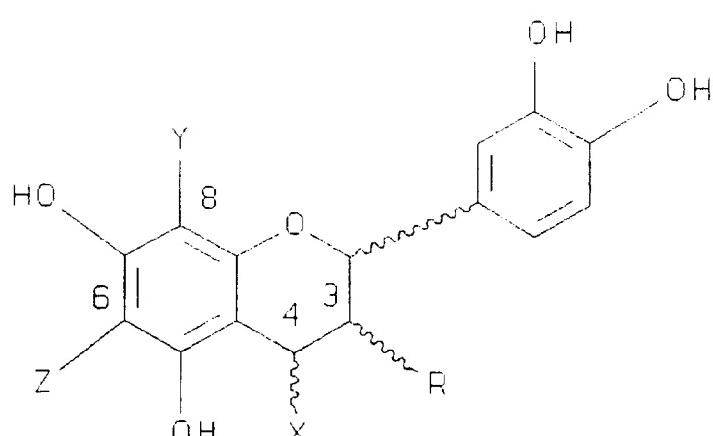
6. The compound of claim 1 which is substantially pure.

7. The compound of claim 1 which is purified to apparent homogeneity.

8. The compound of claim 1 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

9. An antineoplastic composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:

wherein
n is an
integer
from 3
to 18,
such
that
there
is at
least



one terminal monomeric unit A, and a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

10. The composition of claim 9 wherein n is 5 to 12.

11. The composition of claim 9 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

12. The composition of claim 9 wherein said polymeric compound is substantially pure.

13. The composition of claim 9 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

14. The composition of claim 9 wherein n is 5.

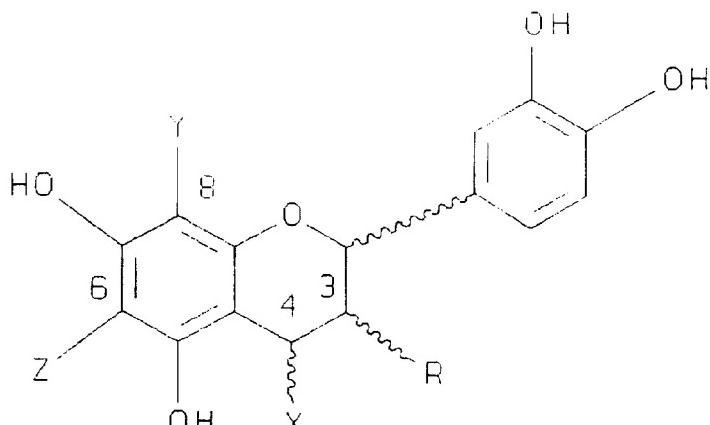
15. A method for treating a subject in need of treatment with an antineoplastic agent comprising administering to the subject an antineoplastic composition as claimed in claim 9.

16. The method of claim 15, further comprising administering at least one additional antineoplastic agent.

17. A kit for a composition of claim 9 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

18. A composition as claimed in claim 9 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

19. An antioxidant composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place
at positions selected from the group consisting of 4, 6
and 8;

a bond of an additional monomeric unit in
position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group
consisting of monomeric unit A, hydrogen, and a sugar,
with the provisos that as to the at least one terminal
monomeric unit, bonding of the additional monomeric unit
thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a
phenolic moiety;

pharmaceutically acceptable salts, derivatives
thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or
excipient.

20. The composition of claim 19 wherein n is 2 to 10.

21. The composition of claim 19 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

22. The composition of claim 19 wherein said polymeric compound is substantially pure.

23. The composition of claim 19 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

24. The composition of claim 19 wherein n is 2 to 12.

25. A method for treating a subject in need of treatment with an antioxidant agent comprising administering to the subject an antioxidant composition as claimed in claim 19.

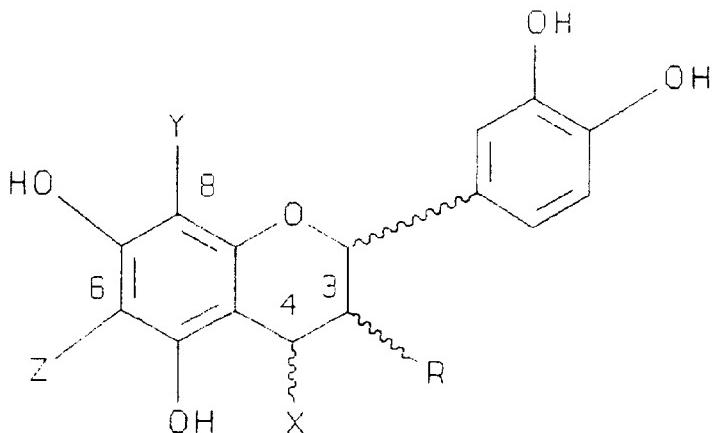
26. The method of claim 25, further comprising administering at least one additional antioxidant agent.

27. A composition as claimed in claim 19 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

28. A kit for a composition of claim 19 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

29. A method for preserving or protecting a desired item from oxidation comprising contacting the item with a composition as claimed in claim 19.

30. An antimicrobial composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

31. The composition of claim 30 wherein n is 2 to 10.

32. The composition of claim 30 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

33. The composition of claim 30 wherein said polymeric compound is substantially pure.

34. The composition of claim 30 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

35. The composition of claim 30 wherein n is selected from the group consisting of 2, 4, 5, 6, 8 and 10.

36. A composition as claimed in claim 30 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

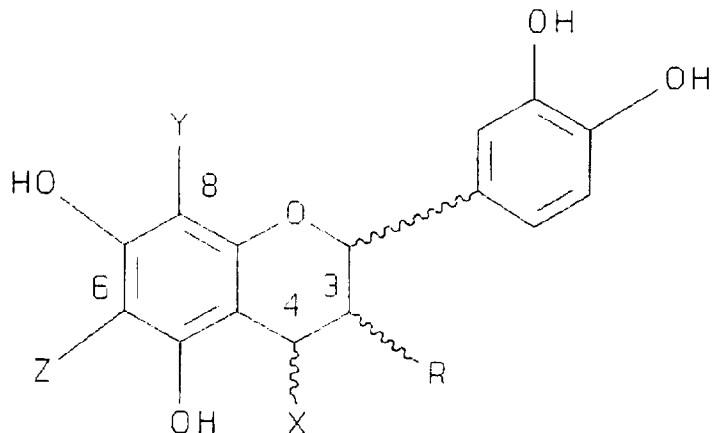
37. A method for treating a subject in need of treatment with an antimicrobial agent comprising administering to the subject an antimicrobial composition as claimed in claim 30.

38. The method of claim 37, further comprising administering at least one additional antimicrobial agent.

39. A kit for a composition of claim 30 comprising the compound and the carrier or diluent

separately packaged, and optionally instructions for admixture or administration.

40. A cyclo-oxygenase and/or lipoxygenase modulating composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is $3-(\alpha)-OH$, $3-(\beta)-OH$, $3-(\alpha)-O-sugar$, or $3-(\beta)-O-sugar$;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

41. The composition of claim 40 wherein n is 2 to 10.

42. The composition of claim 40 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

43. The composition of claim 40 wherein said polymeric compound is substantially pure.

44. The composition of claim 40 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

45. The composition of claim 40 wherein n is 2 to 5.

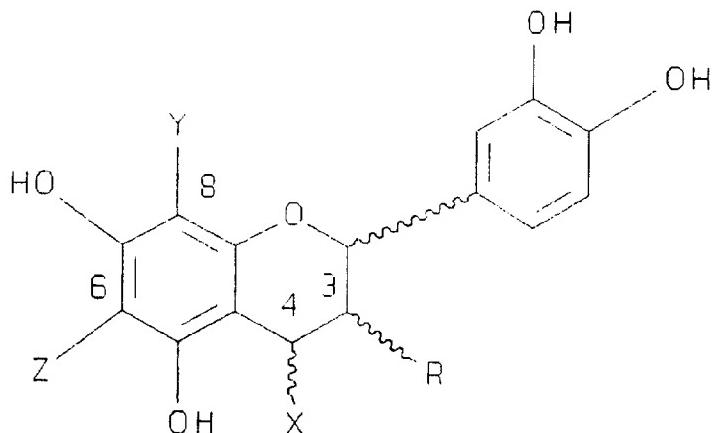
46. A composition as claimed in claim 40 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

47. A method for treating a subject in need of treatment with a cyclo-oxygenase and/or lipoxygenase modulating agent comprising administering to the subject a composition as claimed in claim 40.

48. The method of claim 47, further comprising administering at least one additional cyclo-oxygenase and/or lipoxygenase modulating agent.

49. A kit for a composition of claim 40 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

50. An NO or NO-synthase modulating composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit

thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety via an ester bond;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

51. The composition of claim 50 wherein n is 2 to 10.

52. The composition of claim 50 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

53. The composition of claim 50 wherein said polymeric compound is substantially pure.

54. The composition of claim 50 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

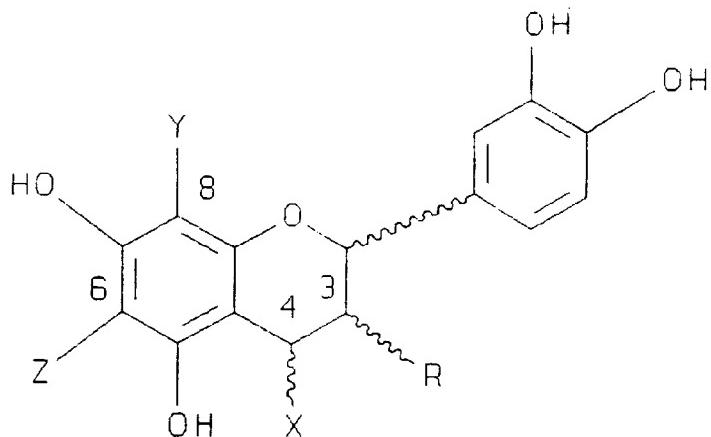
55. A composition as claimed in claim 50 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

56. A method for treating a subject in need of treatment with a NO-modulating agent comprising administering to the subject an NO or NO-synthase modulating composition as claimed in claim 50.

57. The method of claim 56, further comprising administering at least one additional NO or NO-synthase modulating agent.

58. A kit for a composition of claim 50 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

59. A method of treating a mammal in need of treatment with an NO or NO synthase agent comprising administering to the mammal a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar,

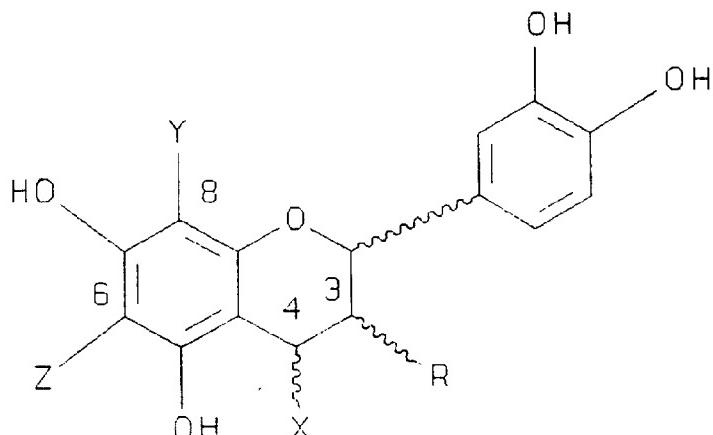
with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

60. A method of reducing NO affected hypercholesterolemia in a mammal comprising administering to said mammal a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

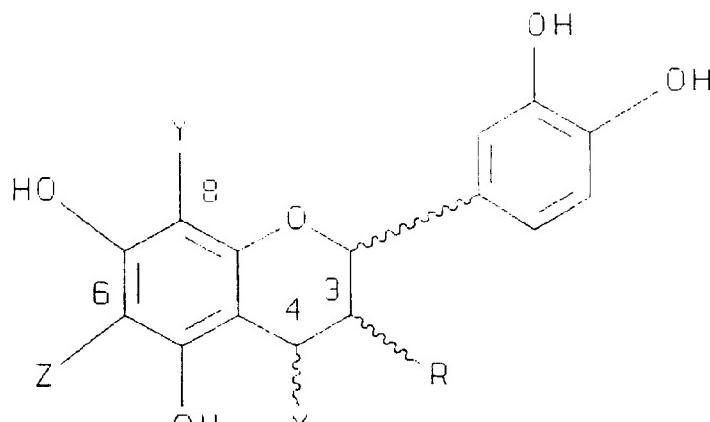
pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

61. The method of claim 60 wherein n is 2 to 10.

62. A method for the induction of iNOS in mammalian monocyte and/or macrophages comprising contacting said monocyte and/or macrophages with a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:

wherein
n is an
integer
from 2
to 18,
such
that
there
is at
least



one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

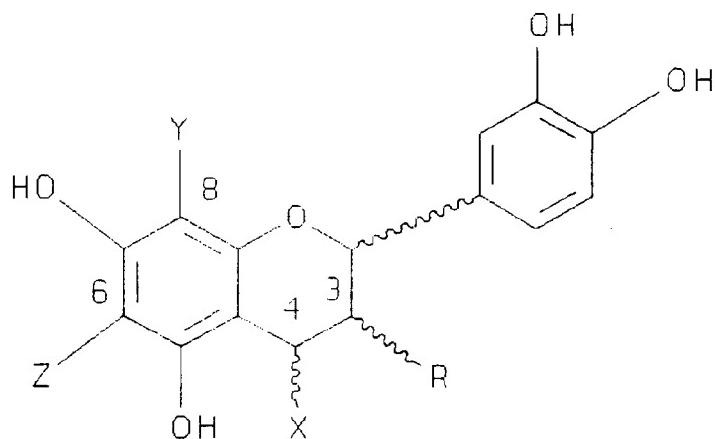
X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

63. A method of stimulating mammalian monocyte and/or macrophage NO production comprising administering to said mammal a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal

monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

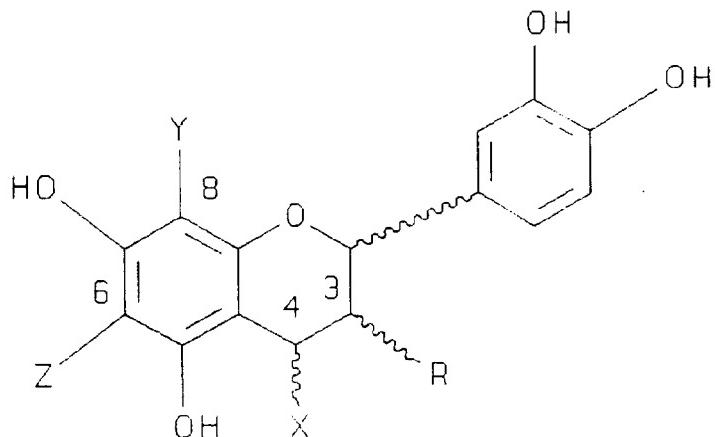
the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

64. The method of claim 63 wherein n is 2 to 10.

65. An *in vivo* glucose-modulating composition comprising a polymeric compound of the formula A_n, wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

66. The composition of claim 65 wherein n is 2 to 10.

67. The composition of claim 65 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

68. The composition of claim 65 wherein said polymeric compound is substantially pure.

69. The composition of claim 65 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

70. A composition as claimed in claim 65 wherein the composition is selected from the group

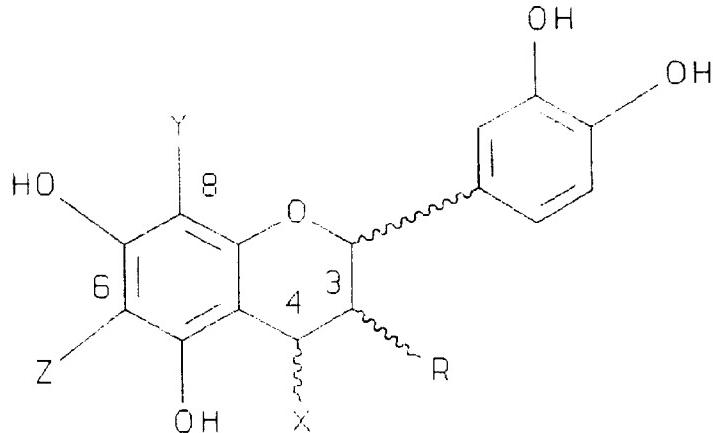
consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

71. A method for treating a subject in need of treatment with a glucose-modulating agent comprising administering to the subject a glucose-modulating agent as claimed in claim 65.

72. The method of claim 71, further comprising administering at least one additional glucose-modulating agent.

73. A kit for a composition of claim 65 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

74. A topoisomerase II-inhibiting composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

75. The composition of claim 74 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

76. The composition of claim 74 wherein said polymeric compound is substantially pure.

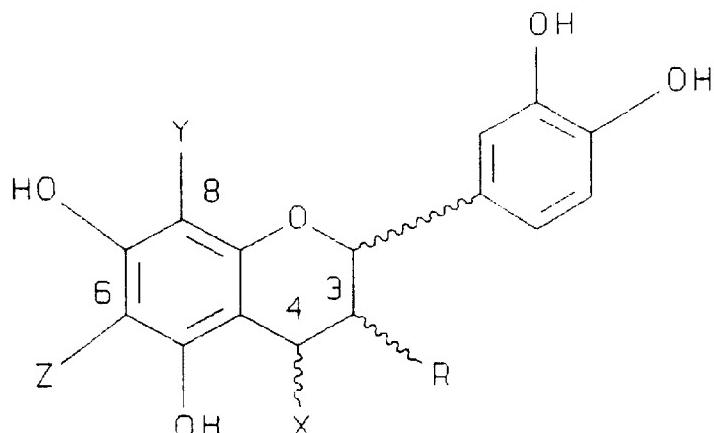
77. The composition of claim 74 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamin, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

78. A composition as claimed in claim 74 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

79. A method for inhibiting topoisomerase II which comprises contacting topoisomerase II with a composition as claimed in claim 74.

80. A kit for a composition of claim 74 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

81. A non-steroidal antiinflammatory composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of the additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of an additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety, and

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

82. The composition of claim 81 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

83. The composition of claim 81 wherein said polymeric compound is substantially pure.

84. The composition of claim 81 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

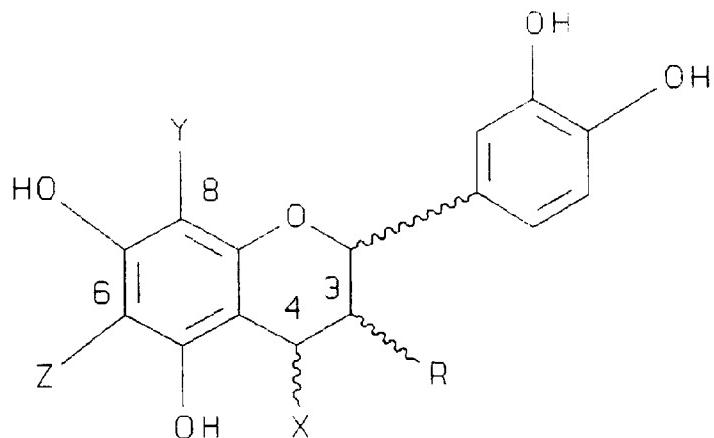
85. A composition as claimed in claim 81 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

86. A method for treating a subject in need of treatment with a non-steroidal antiinflammatory agent comprising administering to the subject a composition as claimed in claim 81.

87. The method of claim 86, further comprising administering at least one additional non-steroidal antiinflammatory agent.

88. A kit for a composition of claim 81 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

89. An anti-gingivitis or anti-periodontitis composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is $3-(\alpha)-OH$, $3-(\beta)-OH$, $3-(\alpha)-O-sugar$, or $3-(\beta)-O-sugar$;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

90. The composition of claim 89 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

91. The composition of claim 89 wherein said polymeric compound is substantially pure.

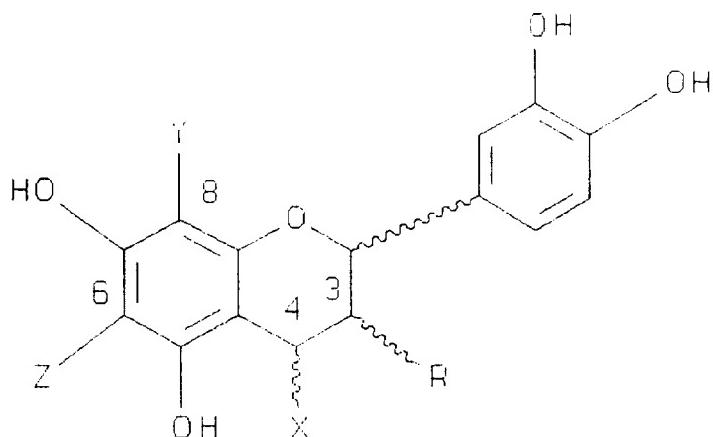
92. The composition of claim 89 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

93. A composition as claimed in claim 89 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

94. A kit for a composition of claim 89 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

95. A method of reducing periodontal disease progression in a mammal comprising administering to said

mammal with a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

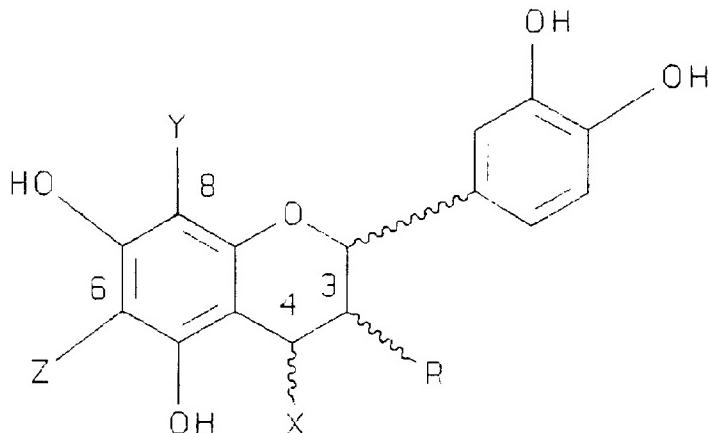
the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

96. The method of claim 95 wherein n is 2 to 10.

97. A method of treatment of periodontal disease comprising administering to a mammal in need of such treatment a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

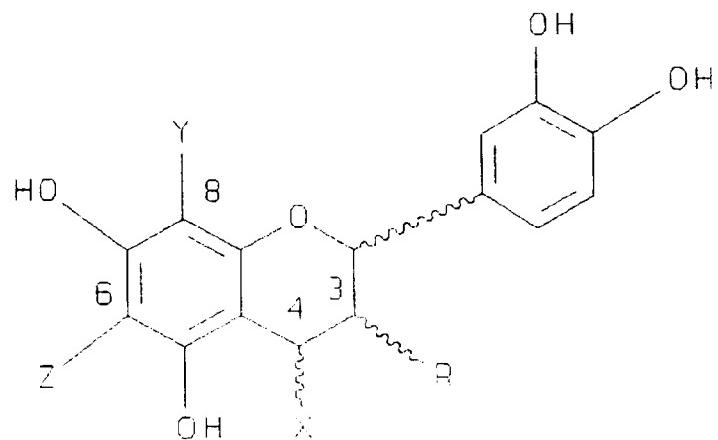
X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

98. A platelet aggregation modulating composition comprising a polymeric compound of the formula A_n, wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

99. The composition of claim 98 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

100. The composition of claim 98 wherein said polymeric compound is substantially pure.

101. The composition of claim 98 wherein the phenolic moiety is selected from the group consisting of

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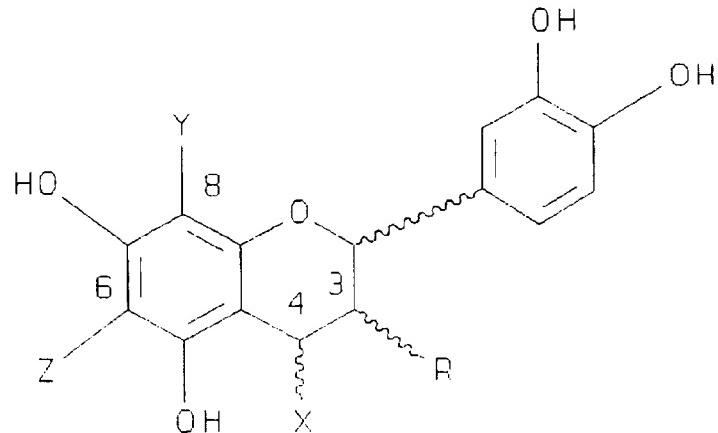
caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

102. A composition as claimed in claim 98 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

103. A kit for a composition of claim 98 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

104. A method of modulating platelet aggregation administering to a mammal in need of such treatment with a compound of claim 98.

105. An apoptosis modulating composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

106. The composition of claim 105 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

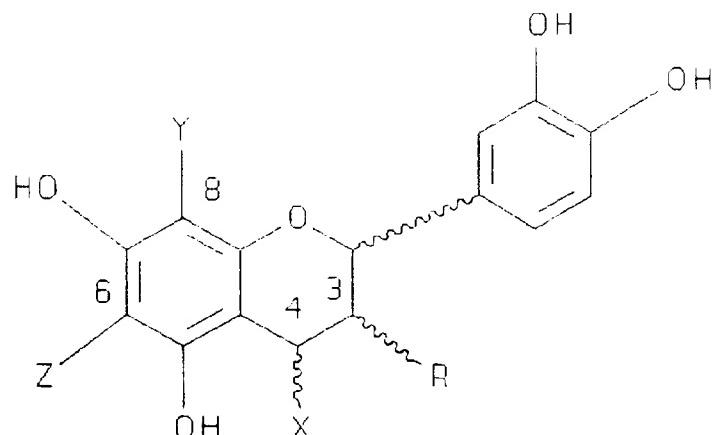
107. The composition of claim 105 wherein said polymeric compound is substantially pure.

108. The composition of claim 105 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

109. A composition as claimed in claim 105 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

110. A kit for a composition of claim 105 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

111. A method of modulating apoptosis comprising contacting COX-2 expressing cancer cells with a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal

monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

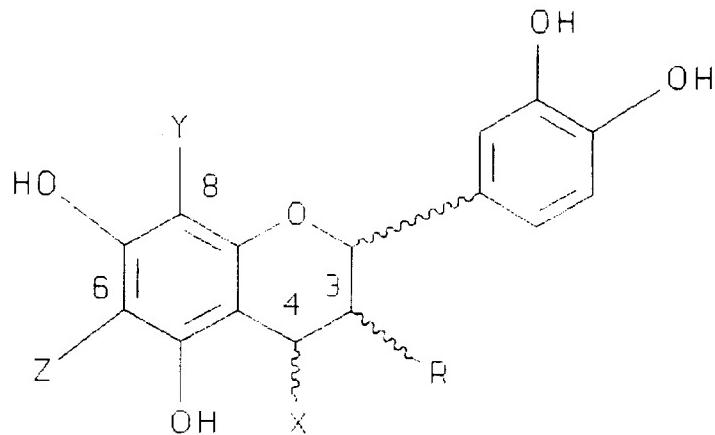
the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

112. The method of claim 111 wherein n is 5.

113. A method of inhibiting oxidative damage to DNA comprising contacting DNA with a composition comprising a polymeric compound of the formula A_n, wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

114. The composition of claim 113 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

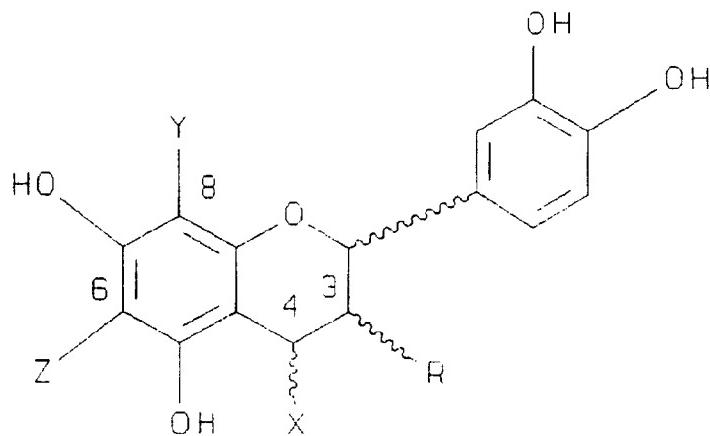
115. The composition of claim 113 wherein said polymeric compound is substantially pure.

116. The composition of claim 113 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

117. A composition as claimed in claim 113 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

118. A kit for a composition of claim 113 comprising the compound and the carrier or diluent separately packaged, and optionally instructions for admixture or administration.

119. A composition for treating, preventing or reducing atherosclerosis or restenosis comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

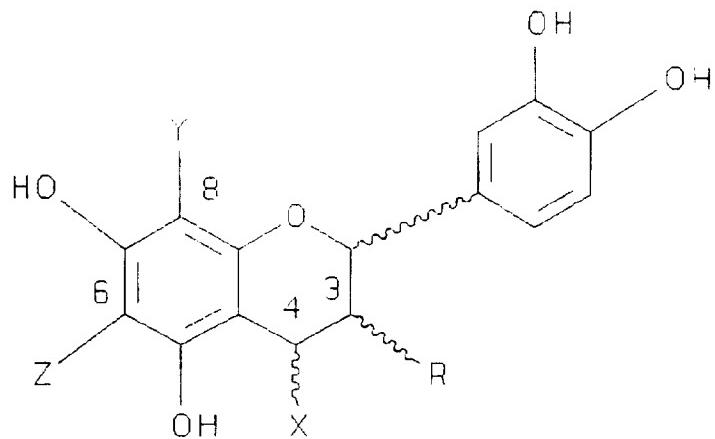
120. The composition of claim 119 wherein the sugar is selected from the group consisting of glucose, galactose, xylose, rhamnose and arabinose.

121. The composition of claim 119 wherein said polymeric compound is substantially pure.

122. The composition of claim 119 wherein the phenolic moiety is selected from the group consisting of caffeic, cinnamic, coumaric, ferulic, gallic, hydroxybenzoic and sinapic acids.

123. A composition as claimed in claim 119 wherein the composition is selected from the group consisting of a tablet, capsule, Standard of Identity chocolate and non-Standard of Identity chocolate.

124. A method of treating, preventing or reducing atherosclerosis or restenosis in a mammal comprising administering to said mammal a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

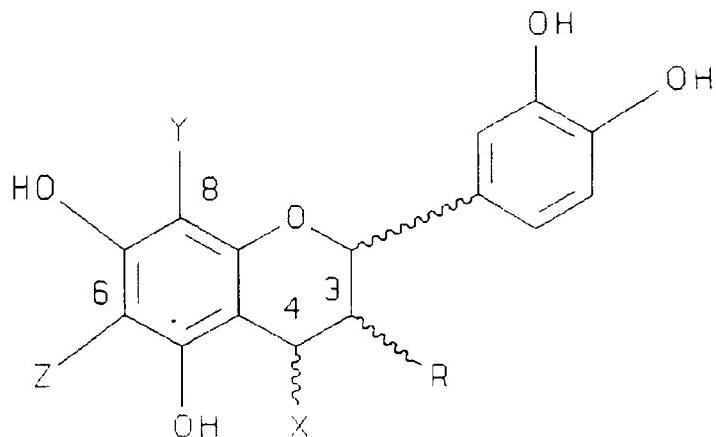
the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

125. The method of claim 124 wherein n is 2 to 10.

126. A method of inhibiting the oxidation of LDL in a mammal comprising administering to said mammal a composition comprising a polymeric compound of the formula A_n, wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

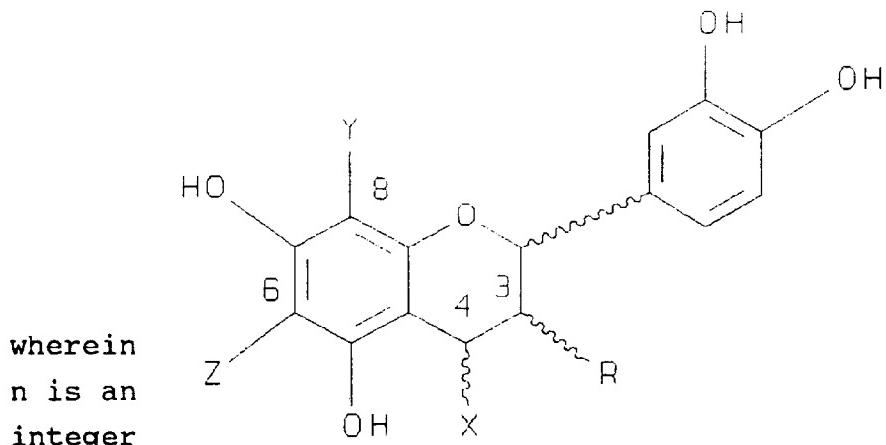
and a pharmaceutically acceptable carrier or excipient.

127. The method of claim 126 wherein n is 2 to 12.

128. A method of modulating thrombosis in a mammal comprising administering to said mammal a compound of claim 1 and a suitable carrier or diluent.

129. The method of claim 128 wherein n is 2 to 12.

130. A method of modulating cyclooxygenase-1 (COX-1) for the treatment for inflammatory bowel disease comprising administering to a mammal in need of such treatment a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place
at positions selected from the group consisting of 4, 6
and 8;

a bond of an additional monomeric unit in
position 4 has alpha or beta stereochemistry;

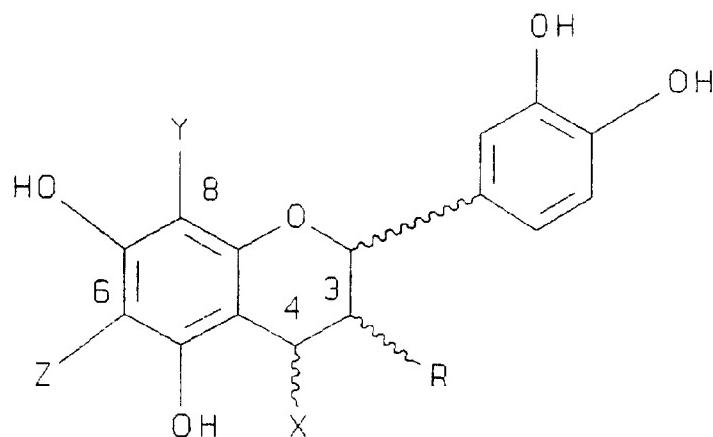
X, Y and Z are selected from the group
consisting of monomeric unit A, hydrogen, and a sugar,
with the provisos that as to the at least one terminal
monomeric unit, bonding of the additional monomeric unit
thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a
phenolic moiety;

pharmaceutically acceptable salts, derivatives
thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or
excipient.

131. A method of inhibiting bacterial growth in a mammal comprising administering to said mammal a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein n is an integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

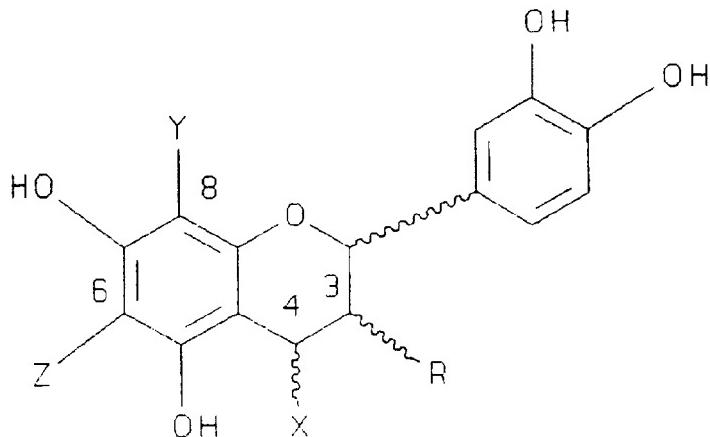
the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

132. The method of claim 131 wherein n is 2, 4, 5, 6, 8 and 10.

133. A method of reducing hypertension in a mammal in need of such treatment comprising administering to said mammal a composition comprising a polymeric compound of the formula A_n , wherein A is a monomer of the formula:



wherein

n is an

integer from 2 to 18, such that there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond of an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety;

pharmaceutically acceptable salts, derivatives thereof, and oxidation products thereof;

and a pharmaceutically acceptable carrier or excipient.

134. The method of claim 133 wherein n is 2 to 10.

135. A method for treating cancer in a mammal in need of such treatment, comprising administering to the mammal in an amount sufficient to effect said treatment of at least one compound in claim 1.

136. The method of claim 135 wherein said cancer is prostate cancer.

137. The method of claim 135 wherein said cancer is renal cancer.

138. The method of claim 135 wherein said cancer is colon cancer.

139. The method of claim 135 wherein said cancer is breast cancer.

140. The method of claim 135 wherein the cancer is feline leukemia.

141. The method of claim 135 wherein the cancer is cervical cancer.

142. The method of claim 135 wherein the cancer is T-cell leukemia.

143. A method of arresting cancer cell growth in a mammal comprising administering to said mammal a compound of claim 1 and a suitable carrier or diluent.

144. The method of claim 143 wherein n is 5.

145. A carrier or vehicle for a pharmaceutical comprising a cocoa extract.

146. A substantially pure polyphenol from *Theobroma* or *Herrania* species or inter- or intra-species specific crosses thereof comprising polyphenols comprising oligomers 3 through 18.

147. A compound of claim 1 having a structure giving an NMR spectra as set forth in Figure 48A-D.

148. A compound of claim 1 having a structure giving an NMR spectra as set forth in Figure 49A-D.

149. A method for enhancing the concentration levels and distribution of cocoa procyanidins in cocoa beans by manipulating fermentation conditions.

150. The compound as claimed in claim 1 wherein the compound is a trimer of formula $[EC-(4\beta\rightarrow8)]_2-EC$.

151. The compound as claimed in claim 1 wherein the compound is a tetramer of formula $[EC-(4\beta\rightarrow8)]_3-EC$.

152. The compound as claimed in claim 1 wherein the compound is a pentamer of formula [EC-(4 β -8)]₄-EC, wherein a 3-position of the terminal monomeric unit of the pentamer is optionally derivatized with a gallate and/or β -D-glucose.

153. The compound as claimed in claim 1 wherein the compound is a hexamer of formula [EC-(4 β -8)]₅-EC.

154. The compound as claimed in claim 1 wherein the compound is a heptamer of formula [EC-(4 β -8)]₆-EC.

155. The compound as claimed in claim 1 wherein the compound is an octamer of formula [EC-(4 β -8)]₇-EC.

156. The compound as claimed in claim 1 wherein the compound is a nonamer of formula [EC-(4 β -8)]₈-EC.

157. The compound as claimed in claim 1 wherein the compound is a decamer of formula [EC-(4 β -8)]₉-EC.

158. The compound as claimed in claim 1 wherein the compound is an undecamer of formula [EC-(4 β -8)]₁₀-EC.

159. The compound as claimed in claim 1 wherein the compound is a dodecamer of formula [EC-(4 β -8)]₁₁-EC.

160. The composition as claimed in claim 9 wherein the compound is selected from the group consisting of a pentamer of formula [EC-(4 β -8)]₄-EC, a hexamer of formula [EC-(4 β -8)]₅-EC, a heptamer of formula [EC-(4 β -8)]₆-EC, an octamer of formula [EC-(4 β -8)]₇-EC, a nonamer of formula [EC-(4 β -8)]₈-EC, a decamer of formula [EC-(4 β -8)]₉-EC, an undecamer of formula [EC-(4 β -8)]₁₀-EC, and a dodecamer of formula [EC-(4 β -8)]₁₁-EC.

161. The composition as claimed in claim 19 wherein the compound is selected from the group consisting of a dimer of formula EC-(4B_→8)-EC, a trimer of formula [EC-(4B_→8)]₂-EC, a tetramer of formula [EC-(4B_→8)]₃-EC, a pentamer of formula [EC-(4B_→8)]₄-EC, a hexamer of formula [EC-(4B_→8)]₅-EC, a heptamer of formula [EC-(4B_→8)]₆-EC, an octamer of formula [EC-(4B_→8)]₇-EC, a nonamer of formula [EC-(4B_→8)]₈-EC, and a decamer of formula [EC-(4B_→8)]₉-EC.

162. The composition as claimed in claim 30 wherein the compound is selected from the group consisting of a dimer of formula EC-(4B_→8)-EC, a trimer of formula [EC-(4B_→8)]₂-EC, a tetramer of formula [EC-(4B_→8)]₃-EC, a pentamer of formula [EC-(4B_→8)]₄-EC, a hexamer of formula [EC-(4B_→8)]₅-EC, a heptamer of formula [EC-(4B_→8)]₆-EC, an octamer of formula [EC-(4B_→8)]₇-EC, a nonamer of formula [EC-(4B_→8)]₈-EC, and a decamer of formula [EC-(4B_→8)]₉-EC.

163. The composition as claimed in claim 40 wherein the compound is selected from the group consisting of a dimer of formula EC-(4B_→8)-EC, a trimer of formula [EC-(4B_→8)]₂-EC, a tetramer of formula [EC-(4B_→8)]₃-EC, a pentamer of formula [EC-(4B_→8)]₄-EC, a hexamer of formula [EC-(4B_→8)]₅-EC, a heptamer of formula [EC-(4B_→8)]₆-EC, an octamer of formula [EC-(4B_→8)]₇-EC, a nonamer of formula [EC-(4B_→8)]₈-EC, and a decamer of formula [EC-(4B_→8)]₉-EC.

164. The composition as claimed in claim 50 wherein the compound is selected from the group consisting of a dimer of formula EC-(4B_→8)-EC, a trimer of formula [EC-(4B_→8)]₂-EC, a tetramer of formula [EC-(4B_→8)]₃-EC, a pentamer of formula [EC-(4B_→8)]₄-EC, a hexamer of formula [EC-(4B_→8)]₅-EC, a heptamer of formula [EC-(4B_→8)]₆-EC, an octamer of formula [EC-(4B_→8)]₇-EC, a nonamer of formula [EC-(4B_→8)]₈-EC, and a decamer of formula [EC-(4B_→8)]₉-EC.

165. The composition as claimed in claim 65 wherein the compound is selected from the group consisting of a dimer of formula EC-(4 β -8)-EC, a trimer of formula [EC-(4 β -8)]₂-EC, a tetramer of formula [EC-(4 β -8)]₃-EC, a pentamer of formula [EC-(4 β -8)]₄-EC, a hexamer of formula [EC-(4 β -8)]₅-EC, a heptamer of formula [EC-(4 β -8)]₆-EC, an octamer of formula [EC-(4 β -8)]₇-EC, a nonamer of formula [EC-(4 β -8)]₈-EC, and a decamer of formula [EC-(4 β -8)]₉-EC.

166. The composition as claimed in claim 74 wherein the compound is selected from the group consisting of a dimer of formula EC-(4 β -8)-EC, a trimer of formula [EC-(4 β -8)]₂-EC, a tetramer of formula [EC-(4 β -8)]₃-EC, a pentamer of formula [EC-(4 β -8)]₄-EC, a hexamer of formula [EC-(4 β -8)]₅-EC, a heptamer of formula [EC-(4 β -8)]₆-EC, an octamer of formula [EC-(4 β -8)]₇-EC, a nonamer of formula [EC-(4 β -8)]₈-EC, a decamer of formula [EC-(4 β -8)]₉-EC, an undecamer of formula [EC-(4 β -8)]₁₀-EC, and a dodecamer of formula [EC-(4 β -8)]₁₁-EC.

167. The composition as claimed in claim 81 wherein the compound is selected from the group consisting of a dimer of formula EC-(4 β -8)-EC, a trimer of formula [EC-(4 β -8)]₂-EC, a tetramer of formula [EC-(4 β -8)]₃-EC, a pentamer of formula [EC-(4 β -8)]₄-EC, a hexamer of formula [EC-(4 β -8)]₅-EC, a heptamer of formula [EC-(4 β -8)]₆-EC, an octamer of formula [EC-(4 β -8)]₇-EC, a nonamer of formula [EC-(4 β -8)]₈-EC, a decamer of formula [EC-(4 β -8)]₉-EC, an undecamer of formula [EC-(4 β -8)]₁₀-EC, and a dodecamer of formula [EC-(4 β -8)]₁₁-EC.

168. The composition as claimed in claim 89 wherein the compound is selected from the group consisting of a dimer of formula EC-(4 β -8)-EC, a trimer of formula [EC-(4 β -8)]₂-EC, a tetramer of formula [EC-(4 β -8)]₃-EC, a pentamer of formula [EC-(4 β -8)]₄-EC, a hexamer of formula [EC-(4 β -8)]₅-EC, a heptamer of formula

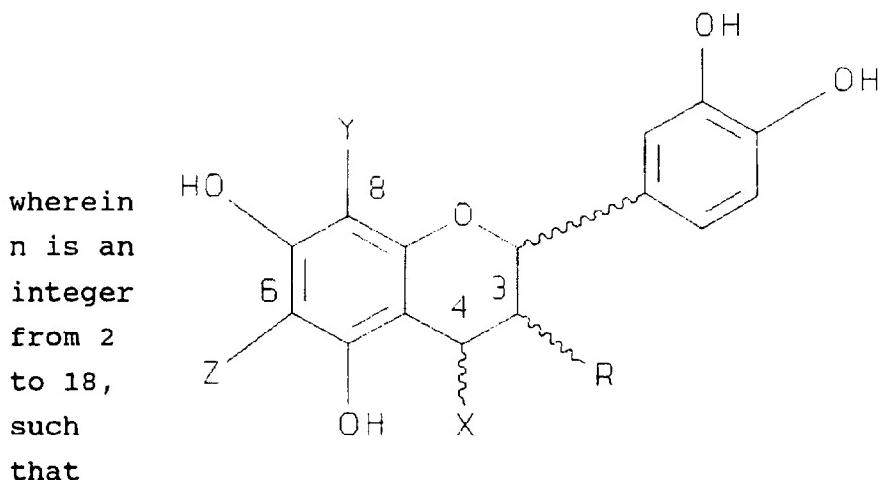
[EC-(4B→8)]₆-EC, an octamer of formula [EC-(4B→8)]₇-EC, a nonamer of formula [EC-(4B→8)]₈-EC, and a decamer of formula [EC-(4B→8)]₉-EC.

169. The composition as claimed in claim 98 wherein the compound is selected from the group consisting of a dimer of formula EC-(4B→8)-EC, a trimer of formula [EC-(4B→8)]₂-EC, a tetramer of formula [EC-(4B→8)]₃-EC, a pentamer of formula [EC-(4B→8)]₄-EC, a hexamer of formula [EC-(4B→8)]₅-EC, a heptamer of formula [EC-(4B→8)]₆-EC, an octamer of formula [EC-(4B→8)]₇-EC, a nonamer of formula [EC-(4B→8)]₈-EC, a decamer of formula [EC-(4B→8)]₉-EC, an undecamer of formula [EC-(4B→8)]₁₀-EC, and a dodecamer of formula [EC-(4B→8)]₁₁-EC.

170. The composition as claimed in claim 105 wherein the compound is a pentamer of formula [EC-(4B→8)]₄-EC.

171. The composition as claimed in claim 119 wherein the compound is selected from the group consisting of a dimer of formula EC-(4B→8)-EC, a trimer of formula [EC-(4B→8)]₂-EC, a tetramer of formula [EC-(4B→8)]₃-EC, a pentamer of formula [EC-(4B→8)]₄-EC, a hexamer of formula [EC-(4B→8)]₅-EC, a heptamer of formula [EC-(4B→8)]₆-EC, an octamer of formula [EC-(4B→8)]₇-EC, a nonamer of formula [EC-(4B→8)]₈-EC, and a decamer of formula [EC-(4B→8)]₉-EC.

172. A method for the identification of at least one gene induced or repressed by a polymeric compound of the formula A_n, wherein A is a monomer of the formula:



there is at least one terminal monomeric unit A, and one or a plurality of additional monomeric units;

R is 3-(α)-OH, 3-(β)-OH, 3-(α)-O-sugar, or 3-(β)-O-sugar;

bonding between adjacent monomers takes place at positions selected from the group consisting of 4, 6 and 8;

a bond for an additional monomeric unit in position 4 has alpha or beta stereochemistry;

X, Y and Z are selected from the group consisting of monomeric unit A, hydrogen, and a sugar, with the provisos that as to the at least one terminal monomeric unit, bonding of the additional monomeric unit thereto is at position 4 and optionally Y = Z = hydrogen;

the sugar is optionally substituted with a phenolic moiety, and

pharmaceutically acceptable salts, derivatives thereof, oxidation products thereof, or combinations thereof,

said method comprising contacting said at least one gene or a gene product thereof with the polymeric compound using a gene expression assay.

173. The method as claimed in claim 172 wherein said gene expression assay is selected from the group consisting of Differential Display, sequencing of cDNA libraries, Serial Analysis of Gene Expression, and expression monitoring by hybridization to high density oligonucleotide arrays.

174. The compound as claimed in claim 1, wherein

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

175. The composition as claimed in claim 9, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

176. The composition as claimed in claim 19,
wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6)
or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

177. The composition as claimed in claim 30,
wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6)
or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

178. The composition as claimed in claim 40,
wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6)
or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent

monomer is at position 4 and optionally, Y = Z = hydrogen.

179. The composition as claimed in claim 50, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

180. The composition as claimed in claim 65, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

181. The composition as claimed in claim 74, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of

the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

182. The composition as claimed in claim 81, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

183. The composition as claimed in claim 89, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

184. The composition as claimed in claim 98, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent

monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

185. The composition as claimed in claim 105, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

186. The composition as claimed in claim 119, wherein the compound further comprises

adjacent monomers bind at position 4 by (4→6) or (4→8); and

each of X, Y and Z is H, a sugar or an adjacent monomer, with the provisos that if X and Y are adjacent monomers, Z is H or sugar and if X and Z are adjacent monomers, Y is H or sugar, and that as to at least one of the two terminal monomers, bonding of the adjacent monomer is at position 4 and optionally, Y = Z = hydrogen.

187. The compound as claimed in claim 1 wherein the compound is a trimer selected from the group consisting of [EC-(4B→8)]₂-EC, [EC-(4B→8)]₂-C and [EC-(4B→6)]₂-EC.

188. The compound as claimed in claim 1 wherein the compound is a tetramer selected from the group

consisting of $[EC-(4\beta\rightarrow 8)]_3-EC$, $[EC-(4\beta\rightarrow 8)]_3-C$ and $[EC-(4\beta\rightarrow 8)]_2-EC-(4\beta\rightarrow 6)-C$.

189. The compound as claimed in claim 1 wherein the compound is a pentamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_4-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 8)-C$ and $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-C$.

190. The compound as claimed in claim 1 wherein the compound is a hexamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_5-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-C$, wherein the 3-position of a hexamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose.

191. The compound as claimed in claim 1 wherein the compound is a heptamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_6-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-C$, wherein the 3-position of the heptamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose.

192. The compound as claimed in claim 1 wherein the compound is an octamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_7-EC$, $[EC-(4\beta\rightarrow 8)]_6-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_6-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_6-EC-(4\beta\rightarrow 6)-C$, wherein the 3-position of an octamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose.

193. The compound as claimed in claim 1 wherein the compound is a nonamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_8-EC$, $[EC-(4\beta\rightarrow 8)]_7-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_7-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_7-EC-(4\beta\rightarrow 6)-C$, wherein the 3-position of a nonamer terminal monomeric

unit is optionally derivatized with a gallate or a β -D-glucose.

194. The compound as claimed in claim 1 wherein the compound is a decamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_9-EC$, $[EC-(4\beta\rightarrow 8)]_8-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_8-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_8-EC-(4\beta\rightarrow 6)-C$, wherein the 3-position of a decamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose.

195. The compound as claimed in claim 1 wherein the compound is an undecamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_{10}-EC$, $[EC-(4\beta\rightarrow 8)]_9-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_9-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_9-EC-(4\beta\rightarrow 6)-C$, wherein the 3-position of an undecamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose.

196. The compound as claimed in claim 1 wherein the compound is a dodecamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_{11}-EC$, $[EC-(4\beta\rightarrow 8)]_{10}-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_{10}-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_{10}-EC-(4\beta\rightarrow 6)-C$, wherein the 3-position of a dodecamer terminal monomeric unit is optionally derivatized with a gallate or a β -D-glucose.

197. The composition as claimed in claim 9 wherein the compound is selected from the group consisting of a pentamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_4-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 8)-C$ and $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-C$, a hexamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_5-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-C$, a heptamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_6-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-C$, an octamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_7-EC$, $[EC-(4\beta\rightarrow 8)]_6-EC-$

(4 β →6)-EC, [EC-(4 β →8)]₆-EC-(4 β →8)-C, and [EC-(4 β →8)]₆-EC-(4 β →6)-C, a nonamer selected from the group consisting of [EC-(4 β →8)]₈-EC, [EC-(4 β →8)]₇-EC-(4 β →6)-EC, [EC-(4 β →8)]₇-EC-(4 β →8)-C, and [EC-(4 β →8)]₇-EC-(4 β →6)-C, a decamer selected from the group consisting of [EC-(4 β →8)]₉-EC, [EC-(4 β →8)]₈-EC-(4 β →6)-EC, [EC-(4 β →8)]₈-EC-(4 β →8)-C, and [EC-(4 β →8)]₈-EC-(4 β →6)-C, an undecamer selected from the group consisting of [EC-(4 β →8)]₁₀-EC, [EC-(4 β →8)]₉-EC-(4 β →6)-EC, [EC-(4 β →8)]₉-EC-(4 β →8)-C, and [EC-(4 β →8)]₉-EC-(4 β →6)-C, and a dodecamer selected from the group consisting of [EC-(4 β →8)]₁₁-EC, [EC-(4 β →8)]₁₀-EC-(4 β →6)-EC, [EC-(4 β →8)]₁₀-EC-(4 β →8)-C, and [EC-(4 β →8)]₁₀-EC-(4 β →6)-C.

198. The composition as claimed in claim 19 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of [EC-(4 β →8)]₂-EC, [EC-(4 β →8)]₂-C and [EC-(4 β →6)]₂-EC, a tetramer selected from the group consisting of [EC-(4 β →8)]₃-EC, [EC-(4 β →8)]₃-C and [EC-(4 β →8)]₂-EC-(4 β →6)-C, a pentamer selected from the group consisting of [EC-(4 β →8)]₄-EC, [EC-(4 β →8)]₃-EC-(4 β →6)-EC, [EC-(4 β →8)]₃-EC-(4 β →8)-C and [EC-(4 β →8)]₃-EC-(4 β →6)-C, a hexamer selected from the group consisting of [EC-(4 β →8)]₅-EC, [EC-(4 β →8)]₄-EC-(4 β →6)-EC, [EC-(4 β →8)]₄-EC-(4 β →8)-C, and [EC-(4 β →8)]₄-EC-(4 β →6)-C, a heptamer selected from the group consisting of [EC-(4 β →8)]₆-EC, [EC-(4 β →8)]₅-EC-(4 β →6)-EC, [EC-(4 β →8)]₅-EC-(4 β →8)-C, and [EC-(4 β →8)]₅-EC-(4 β →6)-C, an octamer selected from the group consisting of [EC-(4 β →8)]₇-EC, [EC-(4 β →8)]₆-EC-(4 β →6)-EC, [EC-(4 β →8)]₆-EC-(4 β →8)-C, and [EC-(4 β →8)]₆-EC-(4 β →6)-C, a nonamer selected from the group consisting of [EC-(4 β →8)]₈-EC, [EC-(4 β →8)]₇-EC-(4 β →6)-EC, [EC-(4 β →8)]₇-EC-(4 β →8)-C, and [EC-(4 β →8)]₇-EC-(4 β →6)-C, and a decamer selected from the group consisting of [EC-(4 β →8)]₉-EC, [EC-(4 β →8)]₈-EC-(4 β →6)-EC, [EC-(4 β →8)]₈-EC-(4 β →8)-C, and [EC-(4 β →8)]₈-EC-(4 β →6)-C.

199. The composition as claimed in claim 30 wherein the compound is selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_2-EC$, $[EC-(4\beta\rightarrow 8)]_2-C$ and $[EC-(4\beta\rightarrow 6)]_2-EC$, a tetramer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_3-EC$, $[EC-(4\beta\rightarrow 8)]_3-C$ and $[EC-(4\beta\rightarrow 8)]_2-EC-(4\beta\rightarrow 6)-C$, a pentamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_4-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 8)-C$ and $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-C$, a hexamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_5-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-C$, a heptamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_6-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-C$, an octamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_7-EC$, $[EC-(4\beta\rightarrow 8)]_6-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_6-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_6-EC-(4\beta\rightarrow 6)-C$, a nonamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_8-EC$, $[EC-(4\beta\rightarrow 8)]_7-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_7-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_7-EC-(4\beta\rightarrow 6)-C$, and a decamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_9-EC$, $[EC-(4\beta\rightarrow 8)]_8-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_8-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_8-EC-(4\beta\rightarrow 6)-C$.

200. The composition as claimed in claim 40 wherein the compound is selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_2-EC$, $[EC-(4\beta\rightarrow 8)]_2-C$ and $[EC-(4\beta\rightarrow 6)]_2-EC$, a tetramer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_3-EC$, $[EC-(4\beta\rightarrow 8)]_3-C$ and $[EC-(4\beta\rightarrow 8)]_2-EC-(4\beta\rightarrow 6)-C$, a pentamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_4-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 8)-C$ and $[EC-(4\beta\rightarrow 8)]_3-EC-(4\beta\rightarrow 6)-C$, a hexamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_5-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_4-EC-(4\beta\rightarrow 6)-C$, a heptamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_6-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-EC$, $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 8)-C$, and $[EC-(4\beta\rightarrow 8)]_5-EC-(4\beta\rightarrow 6)-C$, an

octamer selected from the group consisting of [EC-(4 β →8)]₇-EC, [EC-(4 β →8)]₆-EC-(4 β →6)-EC, [EC-(4 β →8)]₆-EC-(4 β →8)-C, and [EC-(4 β →8)]₆-EC-(4 β →6)-C, a nonamer selected from the group consisting of [EC-(4 β →8)]₈-EC, [EC-(4 β →8)]₇-EC-(4 β →6)-EC, [EC-(4 β →8)]₇-EC-(4 β →8)-C, and [EC-(4 β →8)]₇-EC-(4 β →6)-C, and a decamer selected from the group consisting of [EC-(4 β →8)]₉-EC, [EC-(4 β →8)]₈-EC-(4 β →6)-EC, [EC-(4 β →8)]₈-EC-(4 β →8)-C, and [EC-(4 β →8)]₈-EC-(4 β →6)-C.

201. The composition as claimed in claim 50 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of [EC-(4 β →8)]₂-EC, [EC-(4 β →8)]₂-C and [EC-(4 β →6)]₂-EC, a tetramer selected from the group consisting of [EC-(4 β →8)]₃-EC, [EC-(4 β →8)]₃-C and [EC-(4 β →8)]₂-EC-(4 β →6)-C, a pentamer selected from the group consisting of [EC-(4 β →8)]₄-EC, [EC-(4 β →8)]₃-EC-(4 β →6)-EC, [EC-(4 β →8)]₃-EC-(4 β →8)-C and [EC-(4 β →8)]₃-EC-(4 β →6)-C, a hexamer selected from the group consisting of [EC-(4 β →8)]₅-EC, [EC-(4 β →8)]₄-EC-(4 β →6)-EC, [EC-(4 β →8)]₄-EC-(4 β →8)-C, and [EC-(4 β →8)]₄-EC-(4 β →6)-C, a heptamer selected from the group consisting of [EC-(4 β →8)]₆-EC, [EC-(4 β →8)]₅-EC-(4 β →6)-EC, [EC-(4 β →8)]₅-EC-(4 β →8)-C, and [EC-(4 β →8)]₅-EC-(4 β →6)-C, an octamer selected from the group consisting of [EC-(4 β →8)]₇-EC, [EC-(4 β →8)]₆-EC-(4 β →6)-EC, [EC-(4 β →8)]₆-EC-(4 β →8)-C, and [EC-(4 β →8)]₆-EC-(4 β →6)-C, a nonamer selected from the group consisting of [EC-(4 β →8)]₈-EC, [EC-(4 β →8)]₇-EC-(4 β →6)-EC, [EC-(4 β →8)]₇-EC-(4 β →8)-C, and [EC-(4 β →8)]₇-EC-(4 β →6)-C, and a decamer selected from the group consisting of [EC-(4 β →8)]₉-EC, [EC-(4 β →8)]₈-EC-(4 β →6)-EC, [EC-(4 β →8)]₈-EC-(4 β →8)-C, and [EC-(4 β →8)]₈-EC-(4 β →6)-C.

202. The composition as claimed in claim 65 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of [EC-(4 β →8)]₂-EC, [EC-(4 β →8)]₂-C and [EC-(4 β →6)]₂-EC, a

tetramer selected from the group consisting of [EC-(4B \rightarrow 8)]₃-EC, [EC-(4B \rightarrow 8)]₃-C and [EC-(4B \rightarrow 8)]₂-EC-(4B \rightarrow 6)-C, a pentamer selected from the group consisting of [EC-(4B \rightarrow 8)]₄-EC, [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 8)-C and [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 6)-C, a hexamer selected from the group consisting of [EC-(4B \rightarrow 8)]₅-EC, [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 6)-C, a heptamer selected from the group consisting of [EC-(4B \rightarrow 8)]₆-EC, [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 6)-C, an octamer selected from the group consisting of [EC-(4B \rightarrow 8)]₇-EC, [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 6)-C, a nonamer selected from the group consisting of [EC-(4B \rightarrow 8)]₈-EC, [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 6)-C, and a decamer selected from the group consisting of [EC-(4B \rightarrow 8)]₉-EC, [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 6)-C.

203. The composition as claimed in claim 74 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of [EC-(4B \rightarrow 8)]₂-EC, [EC-(4B \rightarrow 8)]₂-C and [EC-(4B \rightarrow 6)]₂-EC, a tetramer selected from the group consisting of [EC-(4B \rightarrow 8)]₃-EC, [EC-(4B \rightarrow 8)]₃-C and [EC-(4B \rightarrow 8)]₂-EC-(4B \rightarrow 6)-C, a pentamer selected from the group consisting of [EC-(4B \rightarrow 8)]₄-EC, [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 8)-C and [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 6)-C, a hexamer selected from the group consisting of [EC-(4B \rightarrow 8)]₅-EC, [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 6)-C, a heptamer selected from the group consisting of [EC-(4B \rightarrow 8)]₆-EC, [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 6)-C, an octamer selected from the group consisting of [EC-(4B \rightarrow 8)]₇-EC, [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 6)-C, a nonamer selected from the group consisting of [EC-(4B \rightarrow 8)]₈-EC,

[EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 6)-C, a decamer selected from the group consisting of [EC-(4B \rightarrow 8)]₉-EC, [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 6)-C, an undecamer selected from the group consisting of [EC-(4B \rightarrow 8)]₁₀-EC, [EC-(4B \rightarrow 8)]₉-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₉-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₉-EC-(4B \rightarrow 6)-C, and a dodecamer selected from the group consisting of [EC-(4B \rightarrow 8)]₁₁-EC, [EC-(4B \rightarrow 8)]₁₀-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₁₀-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₁₀-EC-(4B \rightarrow 6)-C.

204. The composition as claimed in claim 81 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of [EC-(4B \rightarrow 8)]₂-EC, [EC-(4B \rightarrow 8)]₂-C and [EC-(4B \rightarrow 6)]₂-EC, a tetramer selected from the group consisting of [EC-(4B \rightarrow 8)]₃-EC, [EC-(4B \rightarrow 8)]₃-C and [EC-(4B \rightarrow 8)]₂-EC-(4B \rightarrow 6)-C, a pentamer selected from the group consisting of [EC-(4B \rightarrow 8)]₄-EC, [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 8)-C and [EC-(4B \rightarrow 8)]₃-EC-(4B \rightarrow 6)-C, a hexamer selected from the group consisting of [EC-(4B \rightarrow 8)]₅-EC, [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₄-EC-(4B \rightarrow 6)-C, a heptamer selected from the group consisting of [EC-(4B \rightarrow 8)]₆-EC, [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₅-EC-(4B \rightarrow 6)-C, an octamer selected from the group consisting of [EC-(4B \rightarrow 8)]₇-EC, [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₆-EC-(4B \rightarrow 6)-C, a nonamer selected from the group consisting of [EC-(4B \rightarrow 8)]₈-EC, [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₇-EC-(4B \rightarrow 6)-C, a decamer selected from the group consisting of [EC-(4B \rightarrow 8)]₉-EC, [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₈-EC-(4B \rightarrow 6)-C, an undecamer selected from the group consisting of [EC-(4B \rightarrow 8)]₁₀-EC, [EC-(4B \rightarrow 8)]₉-EC-(4B \rightarrow 6)-EC, [EC-(4B \rightarrow 8)]₉-EC-(4B \rightarrow 8)-C, and [EC-(4B \rightarrow 8)]₉-EC-(4B \rightarrow 6)-C, and a dodecamer selected from the group consisting of [EC-

$(4\beta\rightarrow 8)]_{11}$ -EC, $[EC-(4\beta\rightarrow 8)]_{10}$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_{10}$ -EC- $(4\beta\rightarrow 8)$ -C, and $[EC-(4\beta\rightarrow 8)]_{10}$ -EC- $(4\beta\rightarrow 6)$ -C.

205. The composition as claimed in claim 89 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_2$ -EC, $[EC-(4\beta\rightarrow 8)]_2$ -C and $[EC-(4\beta\rightarrow 6)]_2$ -EC, a tetramer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_3$ -EC, $[EC-(4\beta\rightarrow 8)]_3$ -C and $[EC-(4\beta\rightarrow 8)]_2$ -EC- $(4\beta\rightarrow 6)$ -C, a pentamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_4$ -EC, $[EC-(4\beta\rightarrow 8)]_3$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_3$ -EC- $(4\beta\rightarrow 8)$ -C and $[EC-(4\beta\rightarrow 8)]_3$ -EC- $(4\beta\rightarrow 6)$ -C, a hexamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_5$ -EC, $[EC-(4\beta\rightarrow 8)]_4$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_4$ -EC- $(4\beta\rightarrow 8)$ -C, and $[EC-(4\beta\rightarrow 8)]_4$ -EC- $(4\beta\rightarrow 6)$ -C, a heptamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_6$ -EC, $[EC-(4\beta\rightarrow 8)]_5$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_5$ -EC- $(4\beta\rightarrow 8)$ -C, and $[EC-(4\beta\rightarrow 8)]_5$ -EC- $(4\beta\rightarrow 6)$ -C, an octamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_7$ -EC, $[EC-(4\beta\rightarrow 8)]_6$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_6$ -EC- $(4\beta\rightarrow 8)$ -C, and $[EC-(4\beta\rightarrow 8)]_6$ -EC- $(4\beta\rightarrow 6)$ -C, a nonamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_8$ -EC, $[EC-(4\beta\rightarrow 8)]_7$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_7$ -EC- $(4\beta\rightarrow 8)$ -C, and $[EC-(4\beta\rightarrow 8)]_7$ -EC- $(4\beta\rightarrow 6)$ -C, and a decamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_9$ -EC, $[EC-(4\beta\rightarrow 8)]_8$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_8$ -EC- $(4\beta\rightarrow 8)$ -C, and $[EC-(4\beta\rightarrow 8)]_8$ -EC- $(4\beta\rightarrow 6)$ -C.

206. The composition as claimed in claim 98 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_2$ -EC, $[EC-(4\beta\rightarrow 8)]_2$ -C and $[EC-(4\beta\rightarrow 6)]_2$ -EC, a tetramer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_3$ -EC, $[EC-(4\beta\rightarrow 8)]_3$ -C and $[EC-(4\beta\rightarrow 8)]_2$ -EC- $(4\beta\rightarrow 6)$ -C, a pentamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_4$ -EC, $[EC-(4\beta\rightarrow 8)]_3$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_3$ -EC- $(4\beta\rightarrow 8)$ -C and $[EC-(4\beta\rightarrow 8)]_3$ -EC- $(4\beta\rightarrow 6)$ -C, a hexamer selected from the group consisting of $[EC-(4\beta\rightarrow 8)]_5$ -EC, $[EC-(4\beta\rightarrow 8)]_4$ -EC- $(4\beta\rightarrow 6)$ -EC, $[EC-(4\beta\rightarrow 8)]_4$ -EC- $(4\beta\rightarrow 8)$ -C, and $[EC-(4\beta\rightarrow 8)]_4$ -EC- $(4\beta\rightarrow 6)$ -C.

(4 β -8)]₄-EC-(4 β -6)-C, a heptamer selected from the group consisting of [EC-(4 β -8)]₆-EC, [EC-(4 β -8)]₅-EC-(4 β -6)-EC, [EC-(4 β -8)]₅-EC-(4 β -8)-C, and [EC-(4 β -8)]₅-EC-(4 β -6)-C, an octamer selected from the group consisting of [EC-(4 β -8)]₇-EC, [EC-(4 β -8)]₆-EC-(4 β -6)-EC, [EC-(4 β -8)]₆-EC-(4 β -8)-C, and [EC-(4 β -8)]₆-EC-(4 β -6)-C, a nonamer selected from the group consisting of [EC-(4 β -8)]₈-EC, [EC-(4 β -8)]₇-EC-(4 β -6)-EC, [EC-(4 β -8)]₇-EC-(4 β -8)-C, and [EC-(4 β -8)]₇-EC-(4 β -6)-C, a decamer selected from the group consisting of [EC-(4 β -8)]₉-EC, [EC-(4 β -8)]₈-EC-(4 β -6)-EC, [EC-(4 β -8)]₈-EC-(4 β -8)-C, and [EC-(4 β -8)]₈-EC-(4 β -6)-C, an undecamer selected from the group consisting of [EC-(4 β -8)]₁₀-EC, [EC-(4 β -8)]₉-EC-(4 β -6)-EC, [EC-(4 β -8)]₉-EC-(4 β -8)-C, and [EC-(4 β -8)]₉-EC-(4 β -6)-C, and a dodecamer selected from the group consisting of [EC-(4 β -8)]₁₁-EC, [EC-(4 β -8)]₁₀-EC-(4 β -6)-EC, [EC-(4 β -8)]₁₀-EC-(4 β -8)-C, and [EC-(4 β -8)]₁₀-EC-(4 β -6)-C.

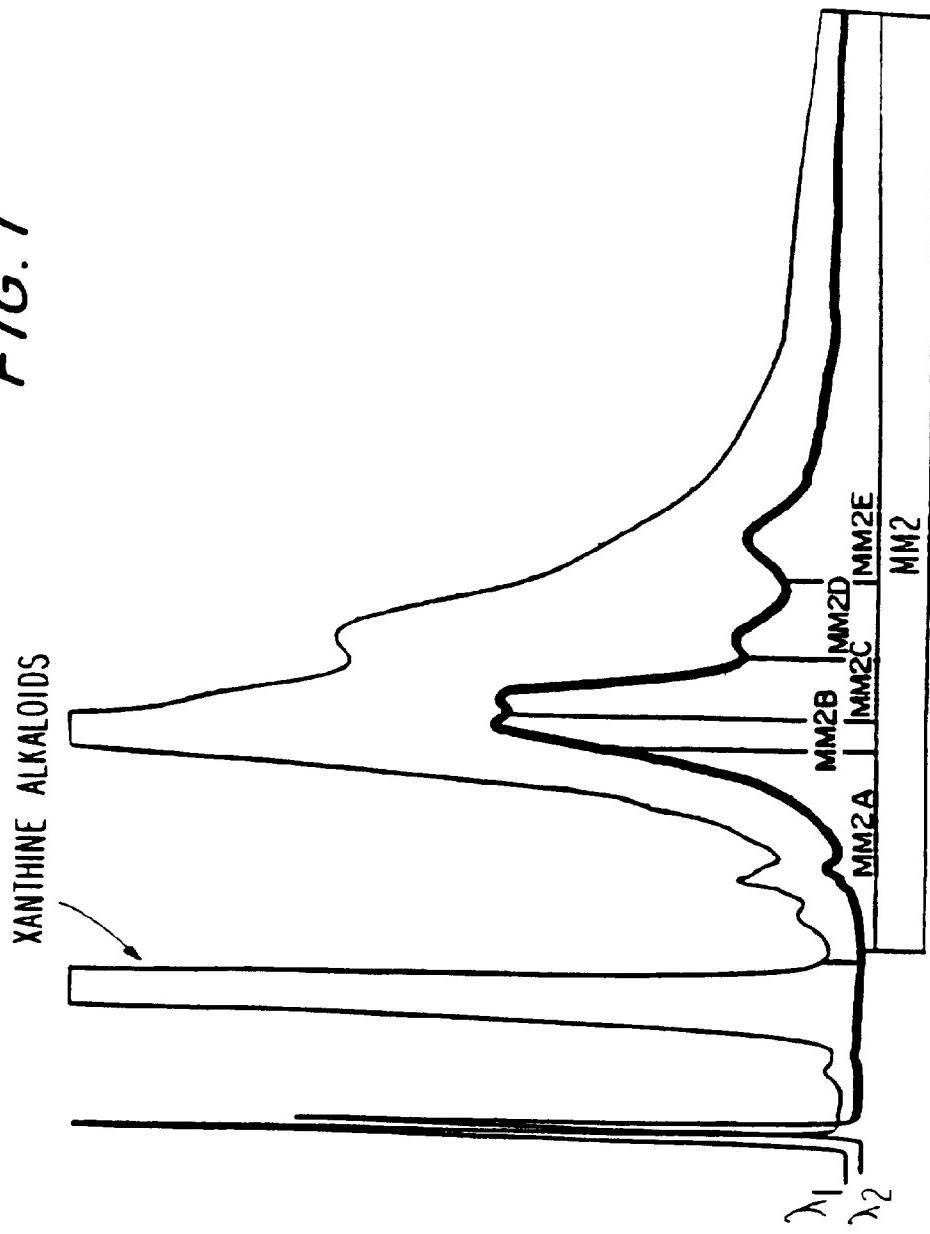
207. The composition as claimed in claim 105 wherein the compound is selected from the group consisting of a pentamer selected from the group consisting of [EC-(4 β -8)]₄-EC, [EC-(4 β -8)]₃-EC-(4 β -6)-EC, [EC-(4 β -8)]₃-EC-(4 β -8)-C and [EC-(4 β -8)]₃-EC-(4 β -6)-C.

208. The composition as claimed in claim 119 wherein the compound is selected from the group consisting of a trimer selected from the group consisting of [EC-(4 β -8)]₂-EC, [EC-(4 β -8)]₂-C and [EC-(4 β -6)]₂-EC, a tetramer selected from the group consisting of [EC-(4 β -8)]₃-EC, [EC-(4 β -8)]₃-C and [EC-(4 β -8)]₂-EC-(4 β -6)-C, a pentamer selected from the group consisting of [EC-(4 β -8)]₄-EC, [EC-(4 β -8)]₃-EC-(4 β -6)-EC, [EC-(4 β -8)]₃-EC-(4 β -8)-C and [EC-(4 β -8)]₃-EC-(4 β -6)-C, a hexamer selected from the group consisting of [EC-(4 β -8)]₅-EC, [EC-(4 β -8)]₄-EC-(4 β -6)-EC, [EC-(4 β -8)]₄-EC-(4 β -8)-C, and [EC-(4 β -8)]₄-EC-(4 β -6)-C, a heptamer selected from the group consisting of [EC-(4 β -8)]₆-EC, [EC-(4 β -8)]₅-EC-(4 β -6)-EC, [EC-(4 β -8)]₅-EC-(4 β -8)-C, and [EC-(4 β -8)]₅-EC-(4 β -6)-C, an

octamer selected from the group consisting of [EC-(4 β →8)]₇-EC, [EC-(4 β →8)]₆-EC-(4 β →6)-EC, [EC-(4 β →8)]₆-EC-(4 β →8)-C, and [EC-(4 β →8)]₆-EC-(4 β →6)-C, a nonamer selected from the group consisting of [EC-(4 β →8)]₈-EC, [EC-(4 β →8)]₇-EC-(4 β →6)-EC, [EC-(4 β →8)]₇-EC-(4 β →8)-C, and [EC-(4 β →8)]₇-EC-(4 β →6)-C, and a decamer selected from the group consisting of [EC-(4 β →8)]₉-EC, [EC-(4 β →8)]₈-EC-(4 β →6)-EC, [EC-(4 β →8)]₈-EC-(4 β →8)-C, and [EC-(4 β →8)]₈-EC-(4 β →6)-C.

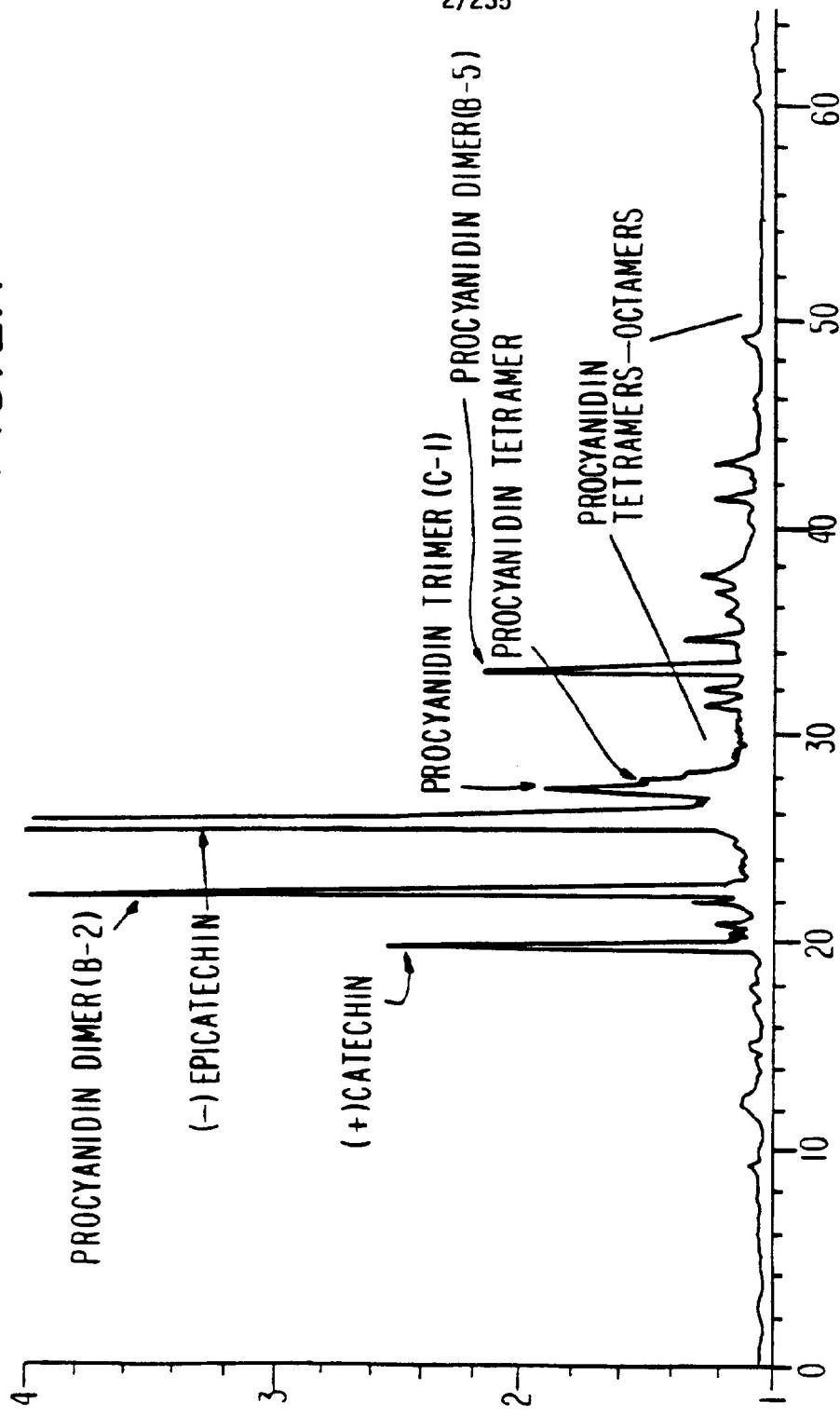
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FIG. 1



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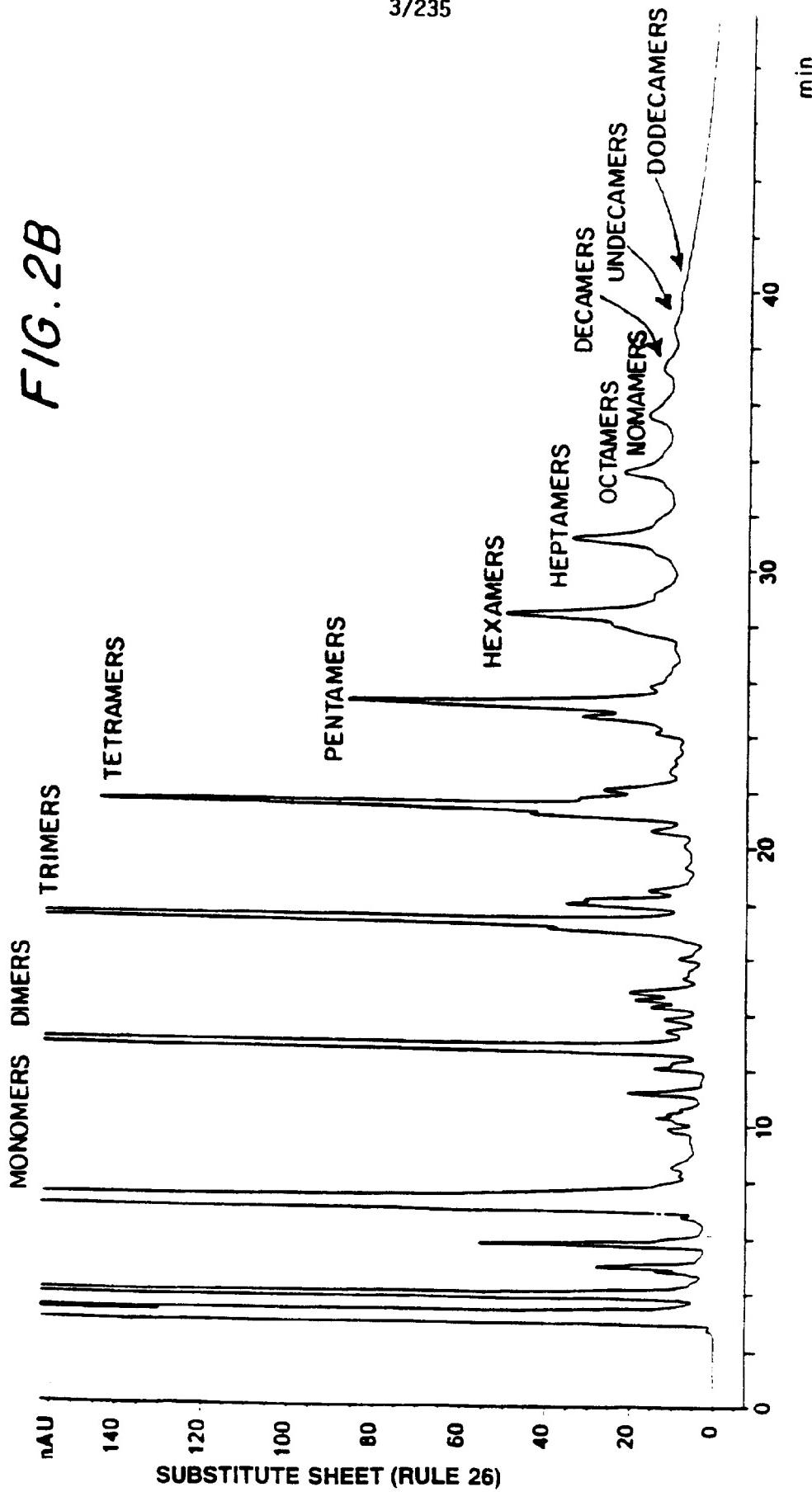
FIG. 2A



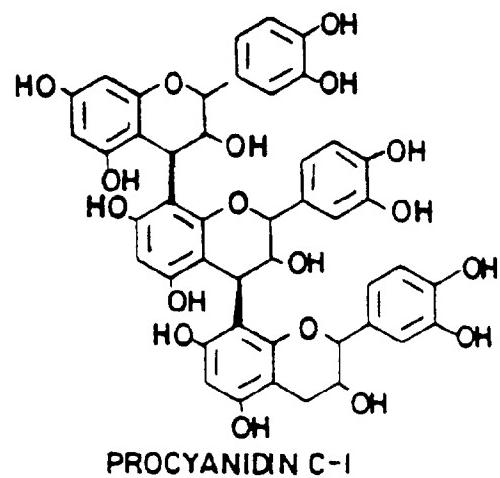
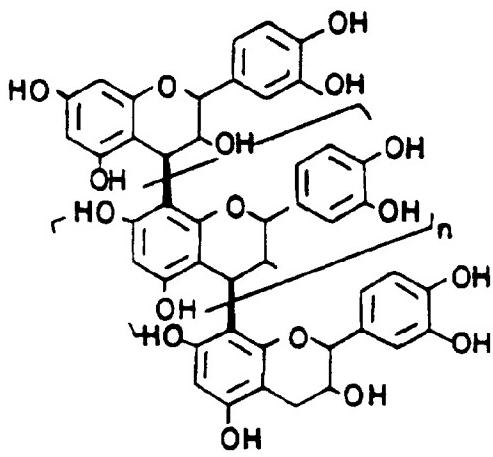
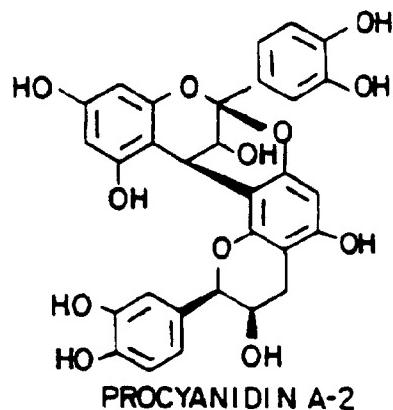
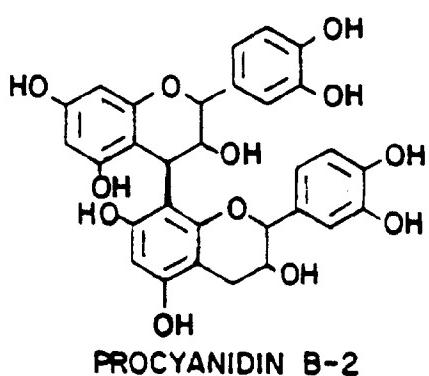
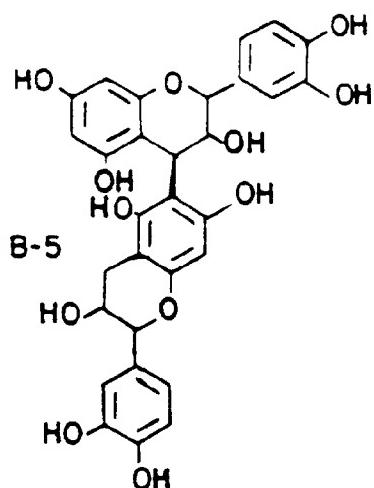
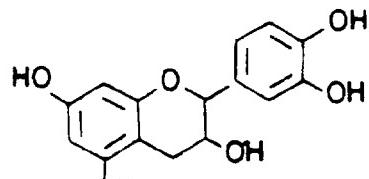
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FIG. 2B

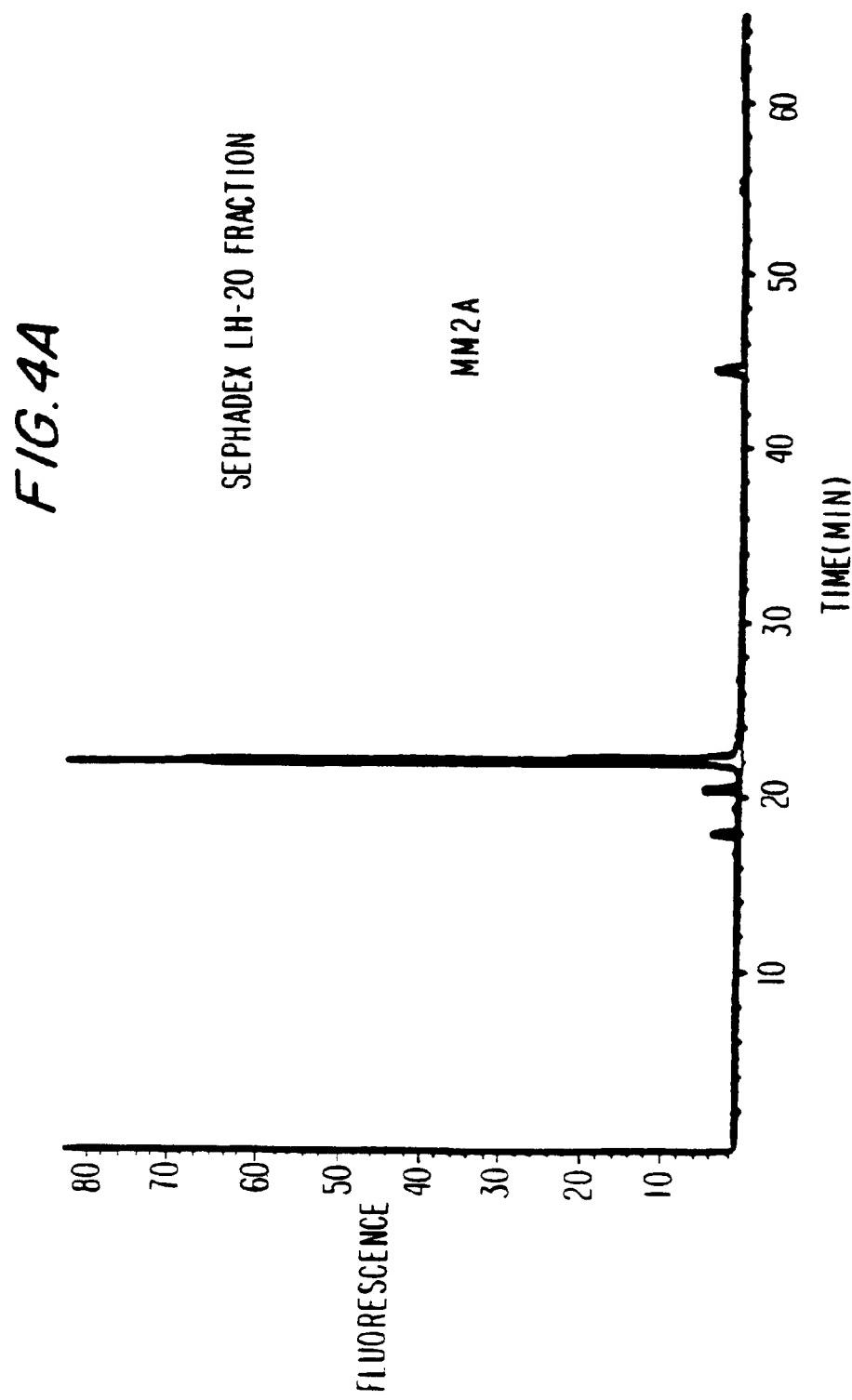
DADI A, Sig > 280, 4 Ref=580, 40d4 4078/009-0401.D



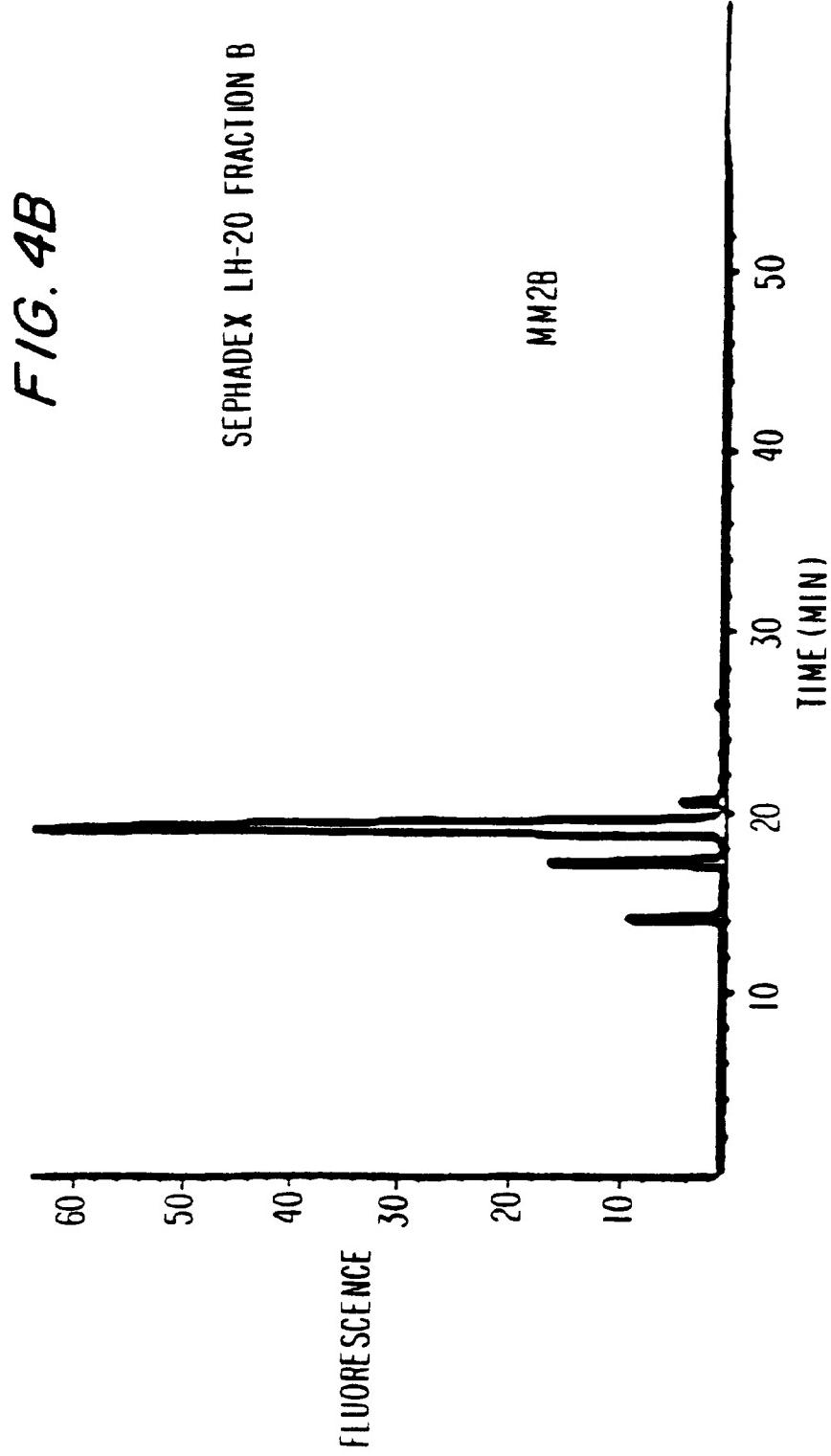
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*FIG. 3*

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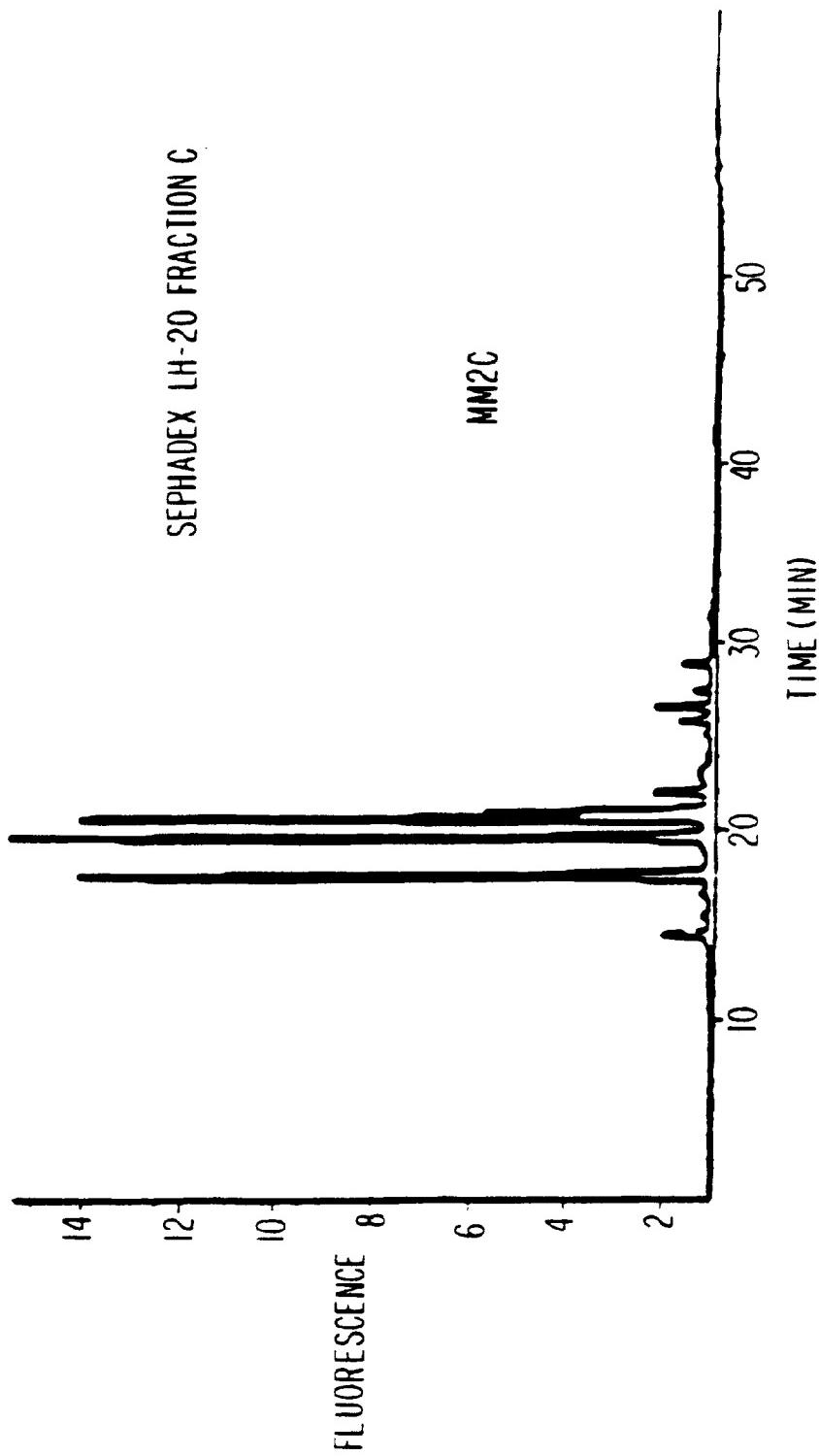


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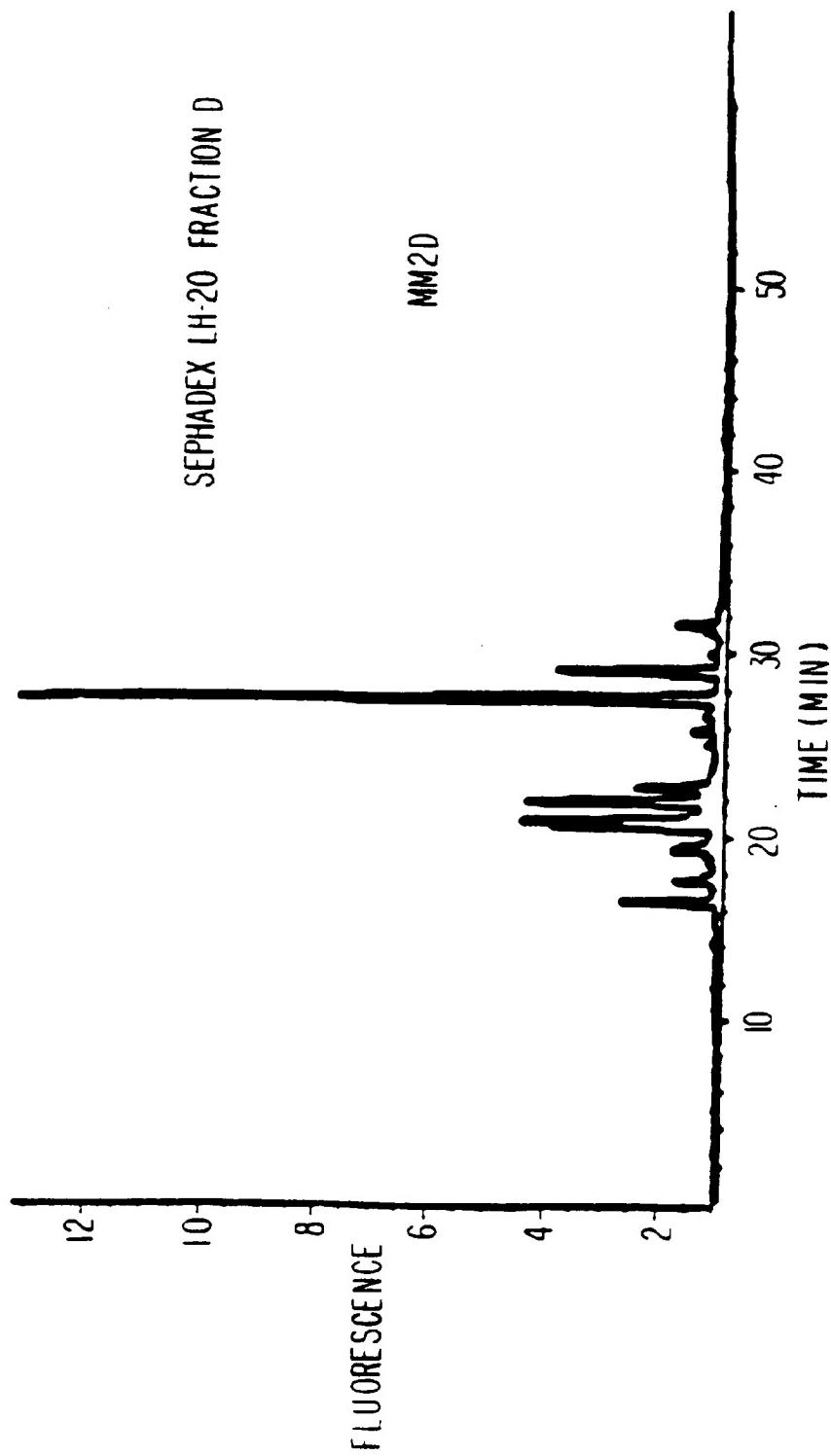
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FIG. 4C

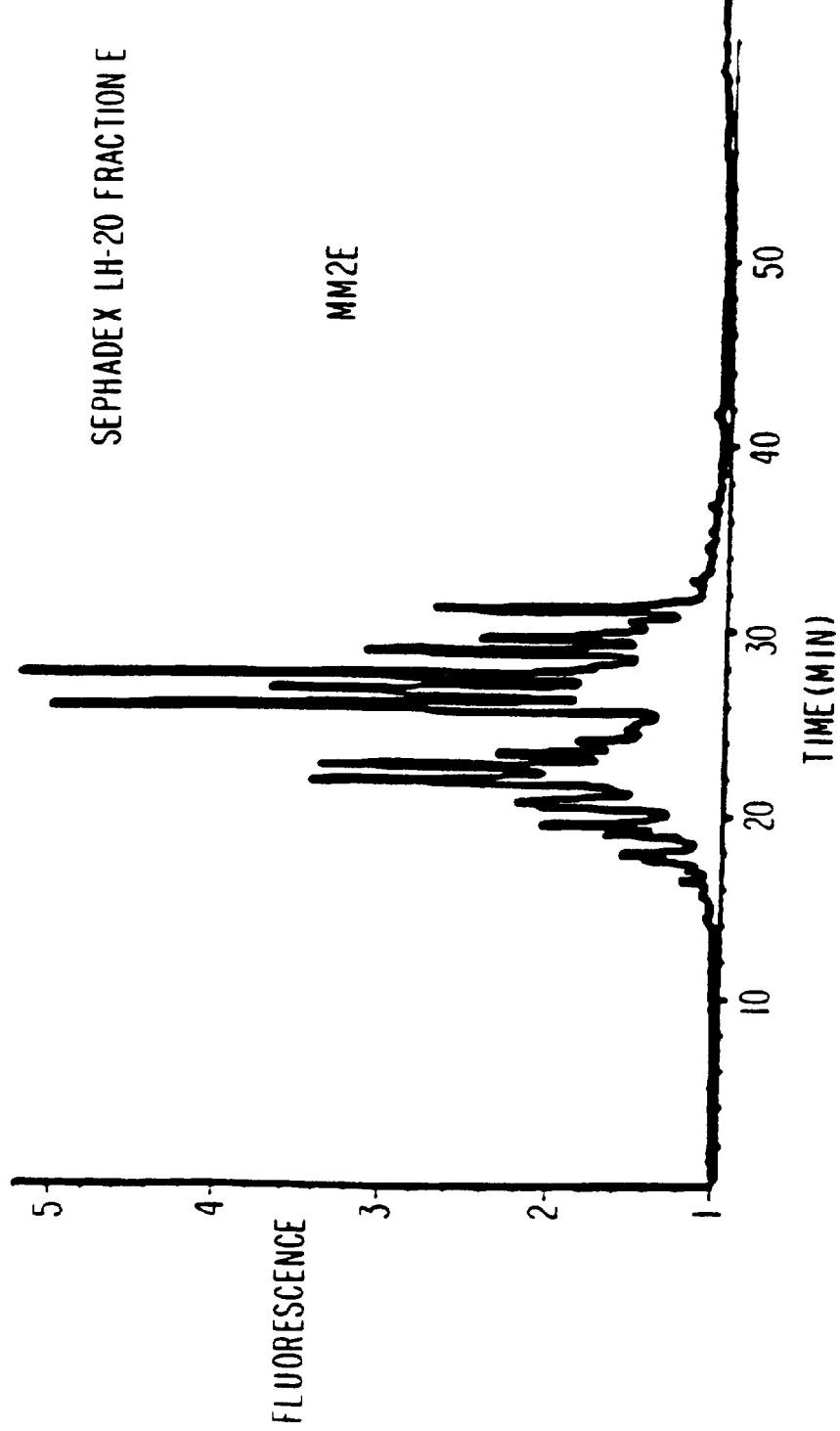


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FIG. 4D

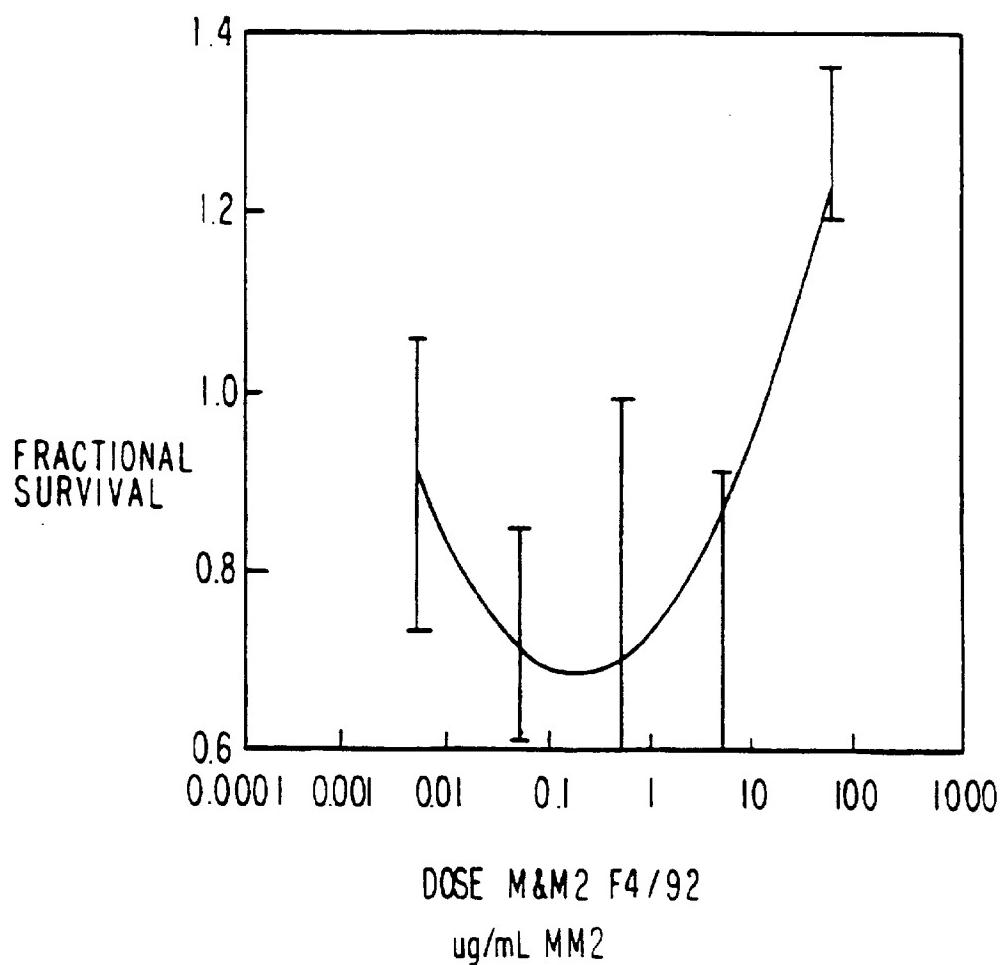


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FIG. 4E

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FIG. 5



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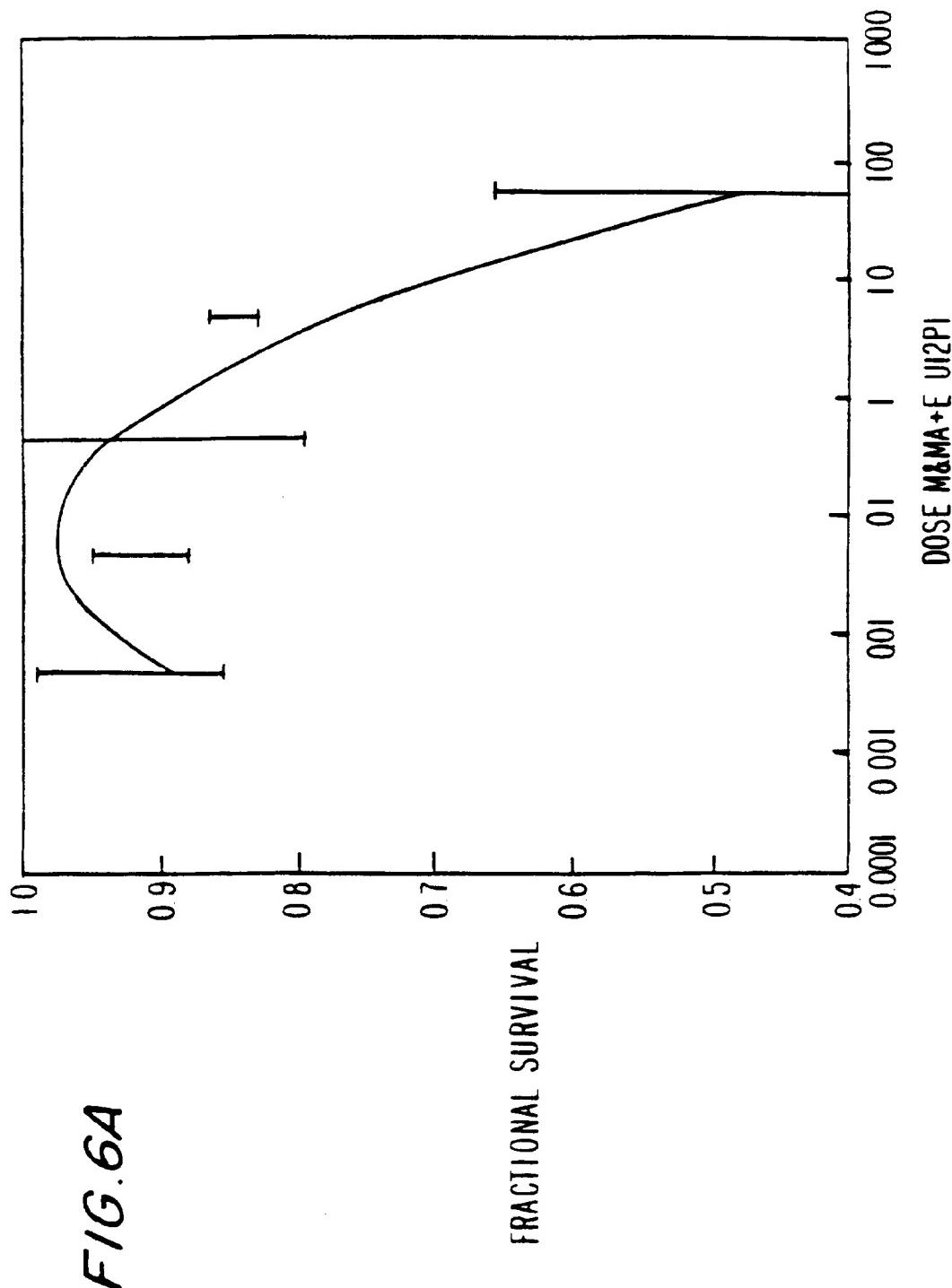
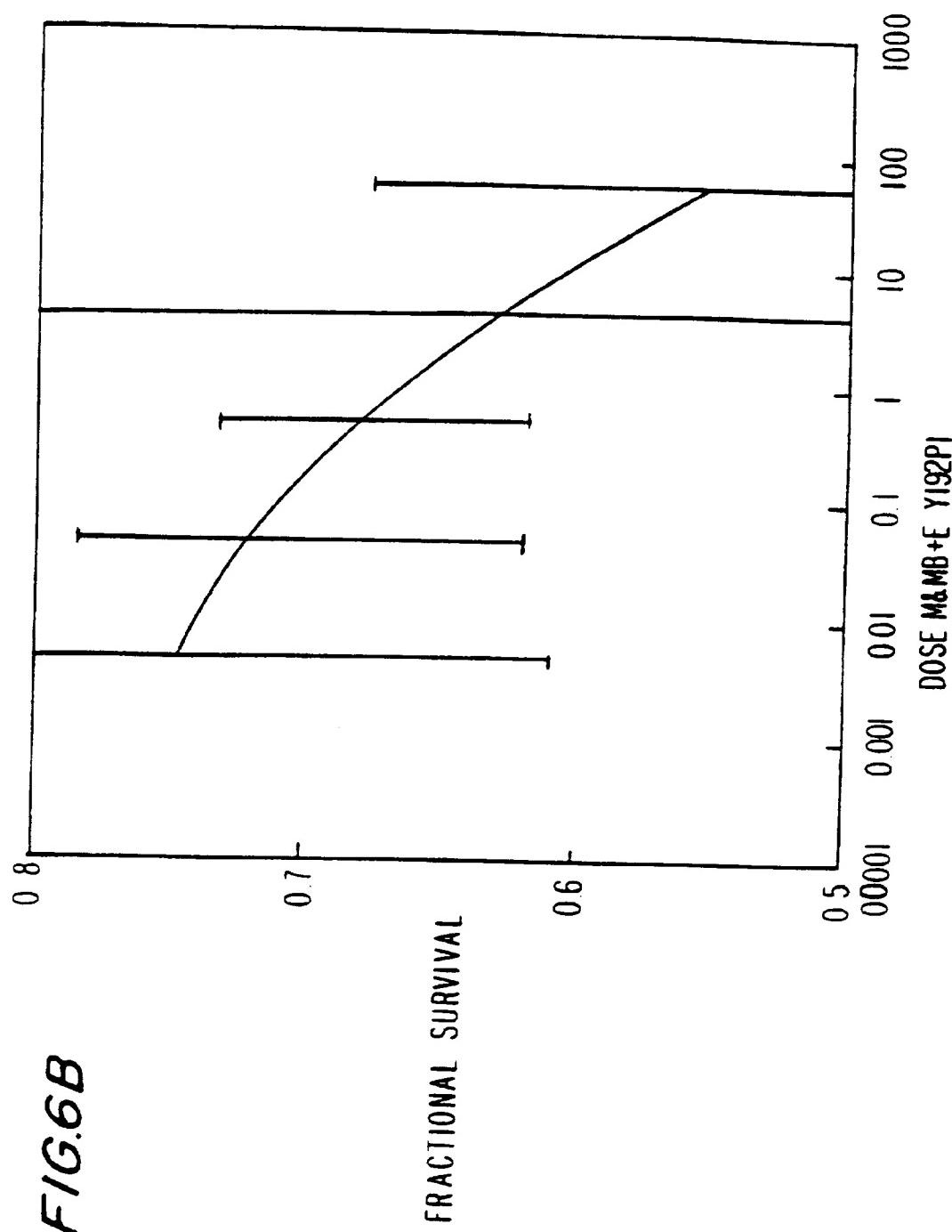


FIG. 6A

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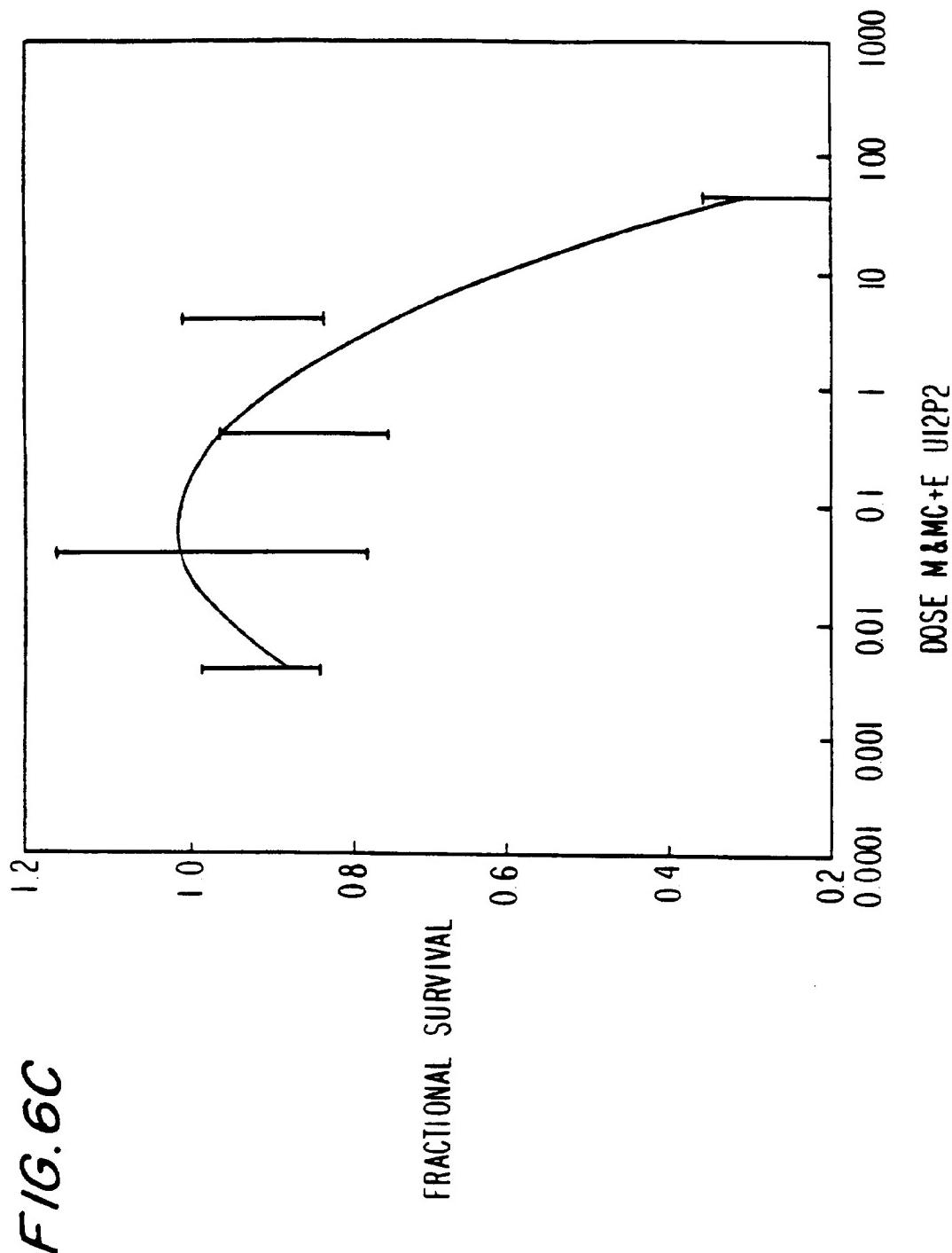


FIG. 6C

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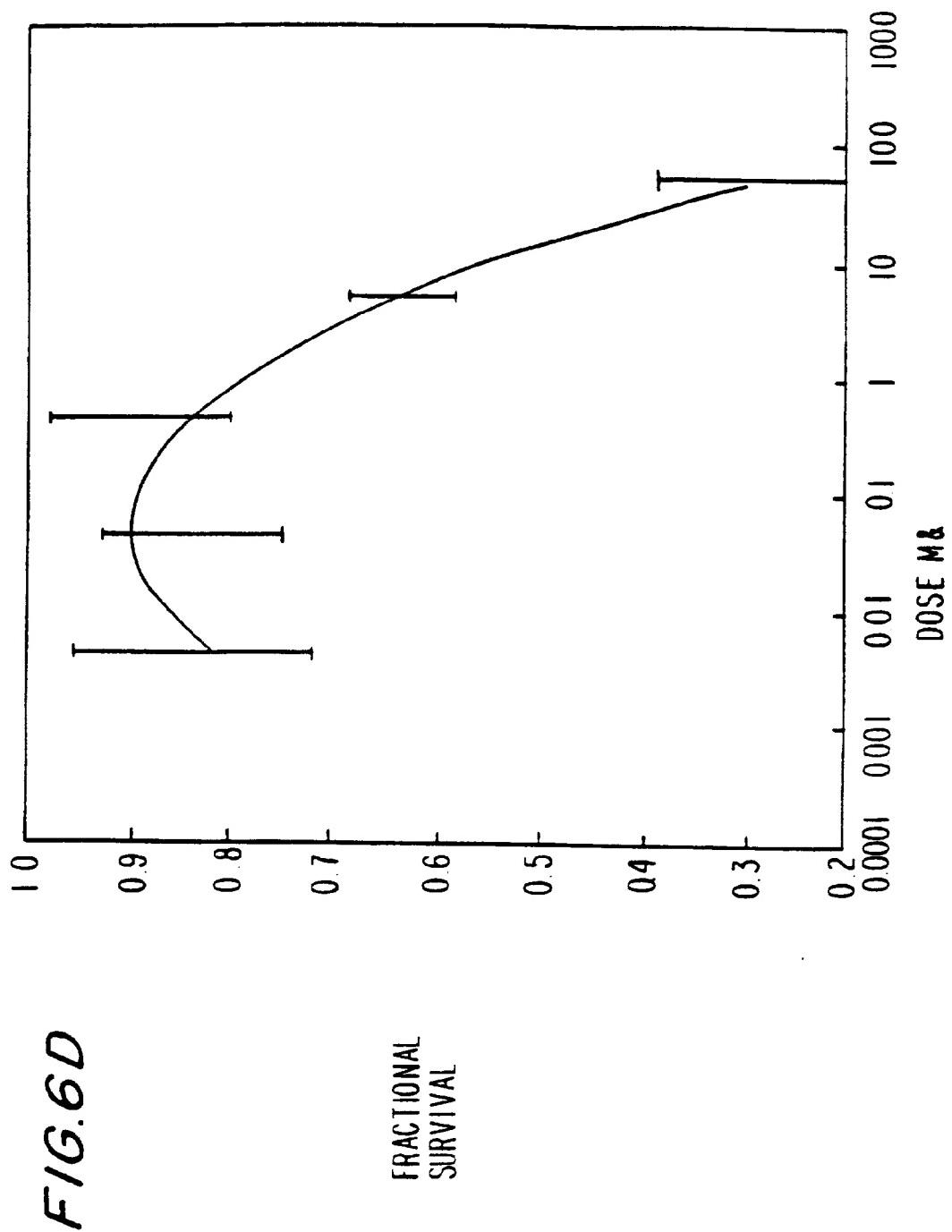
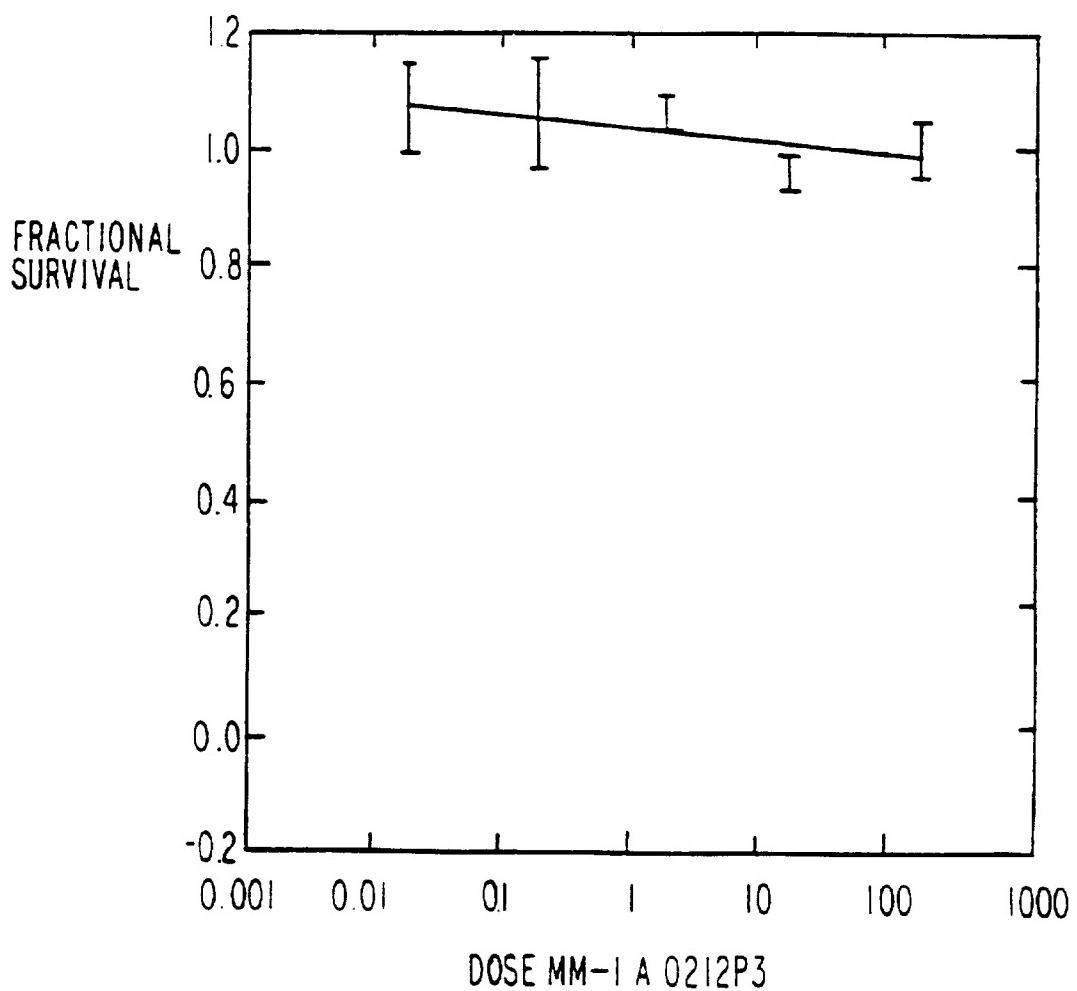


FIG. 6D

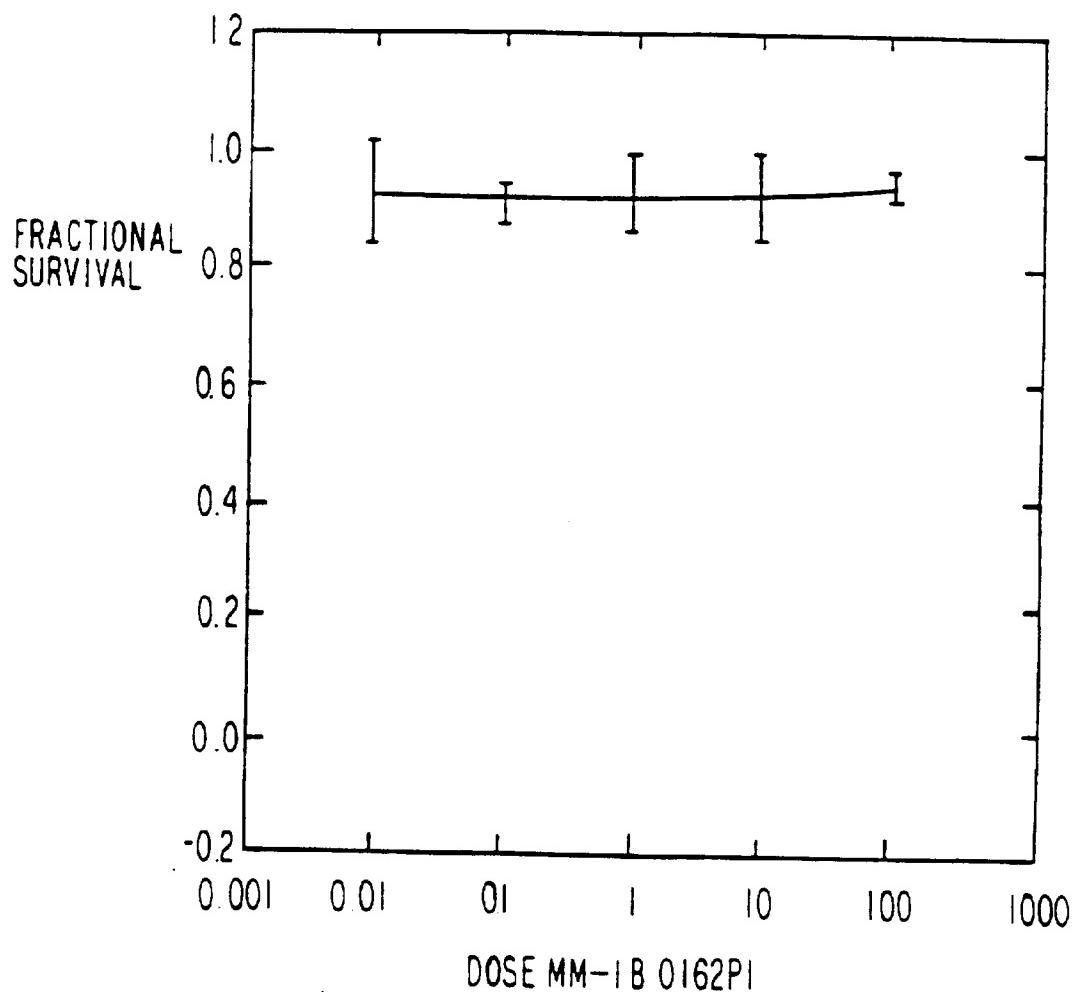
FRACTIONAL
SURVIVAL

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FIG. 7A

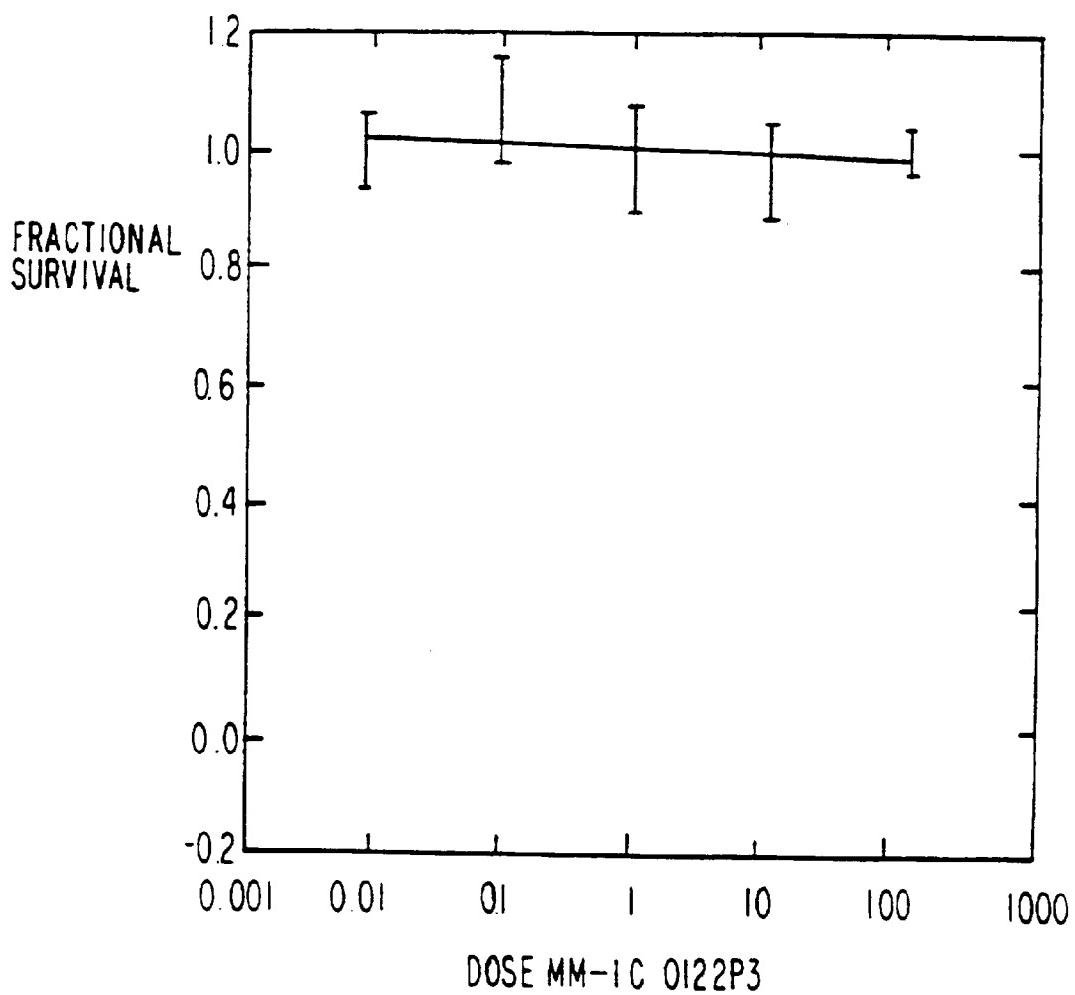


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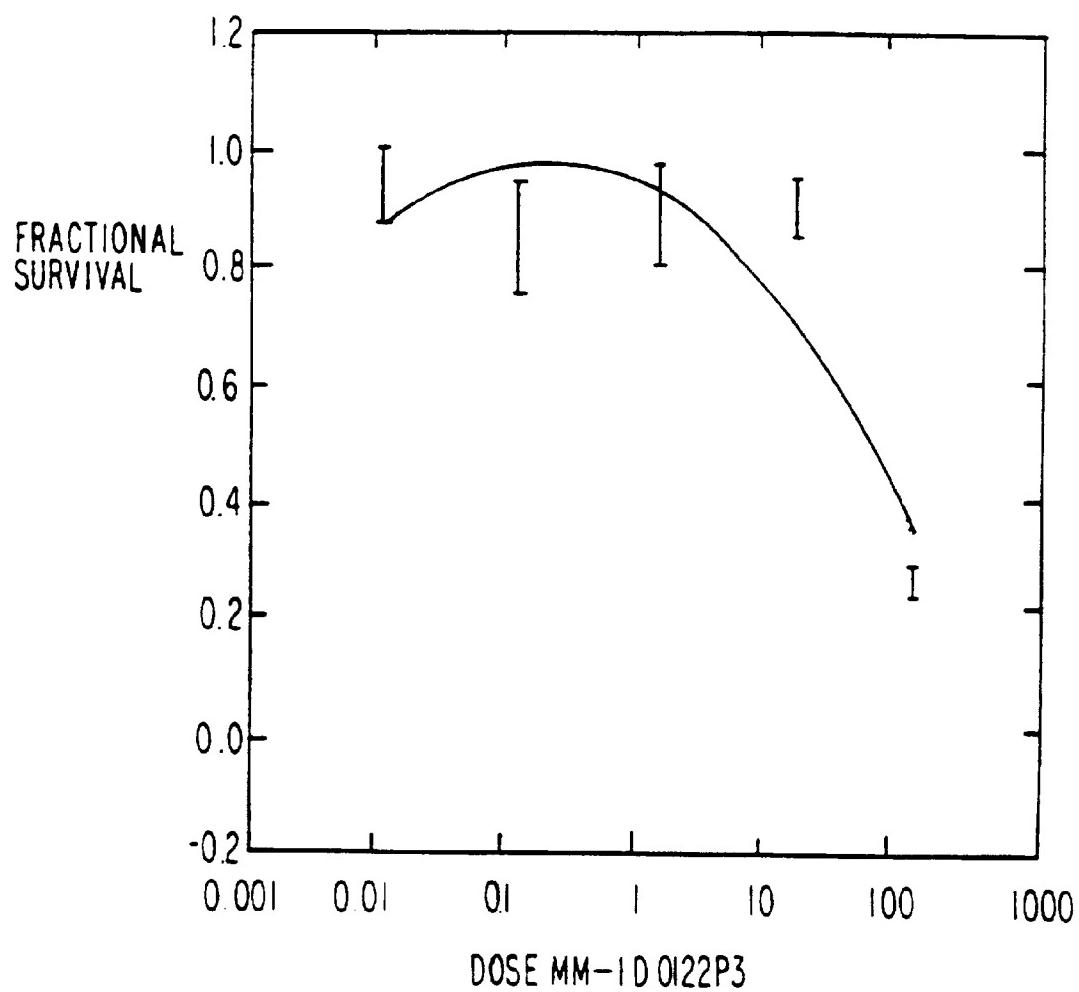
FIG. 7B

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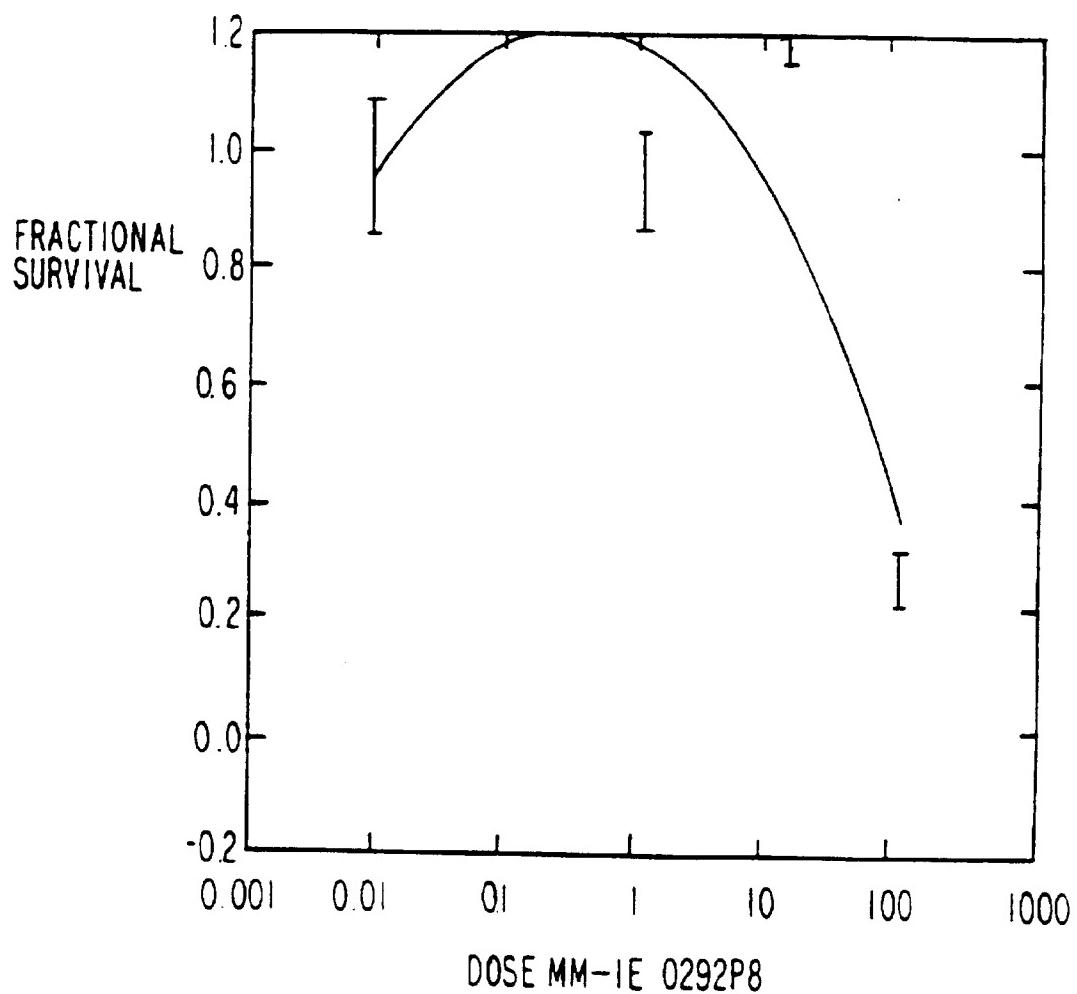
FIG. 7C



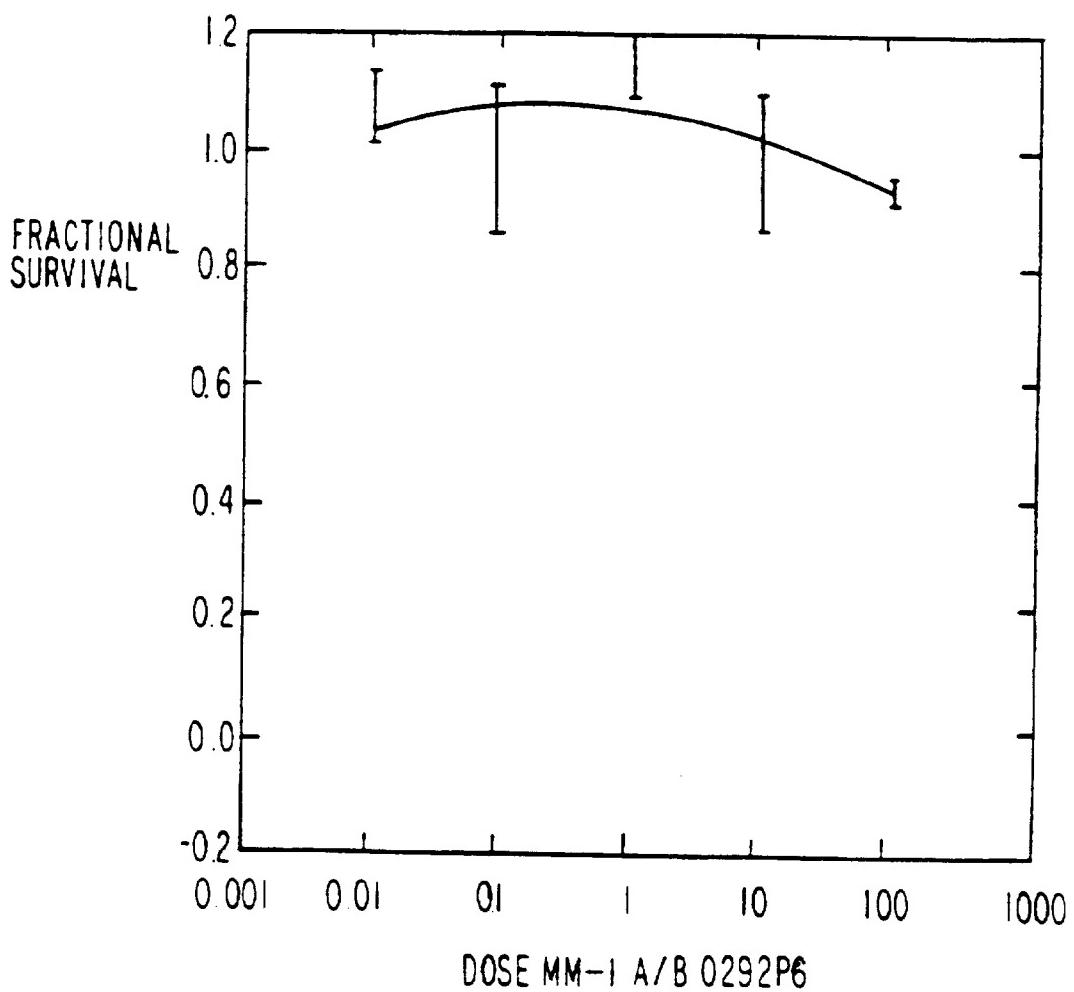
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FIG. 7D

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FIG. 7E

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FIG. 7F

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FIG. 7G

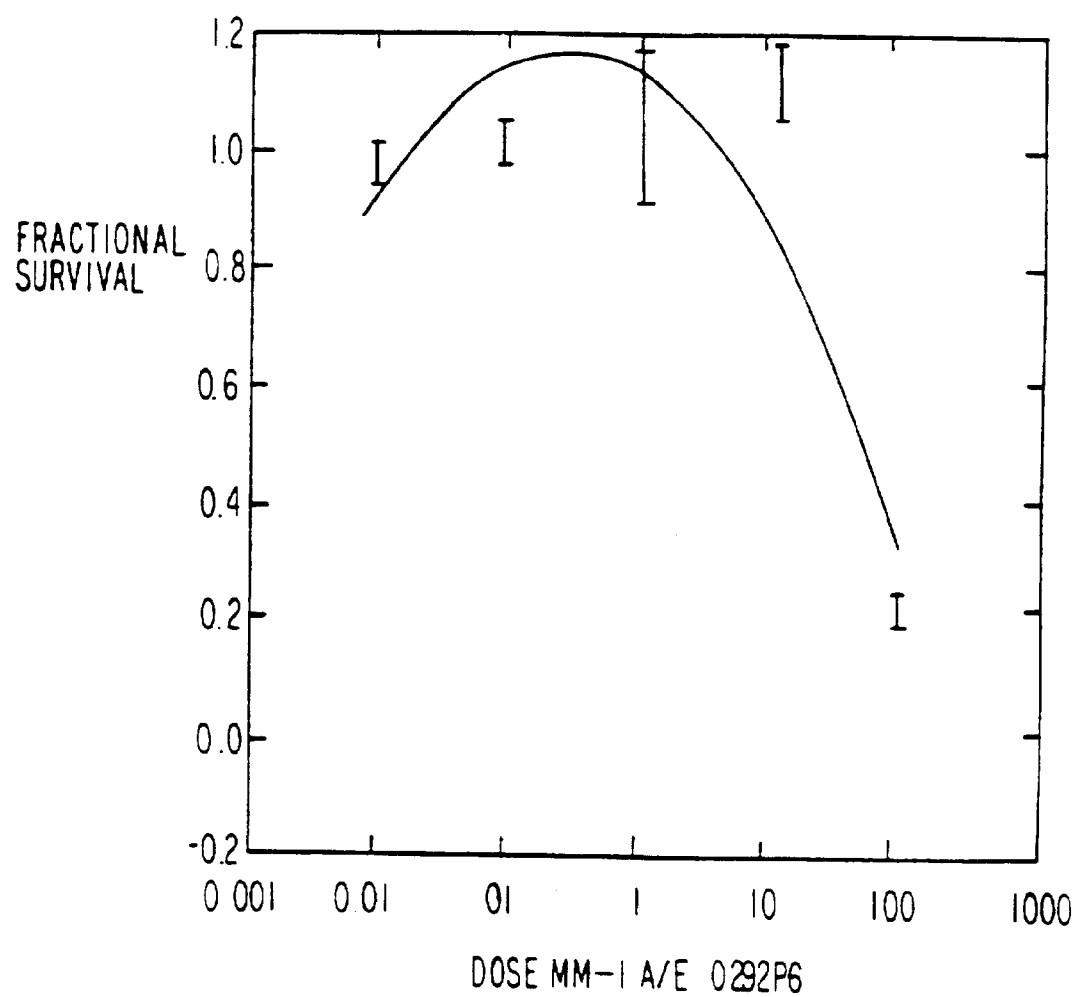
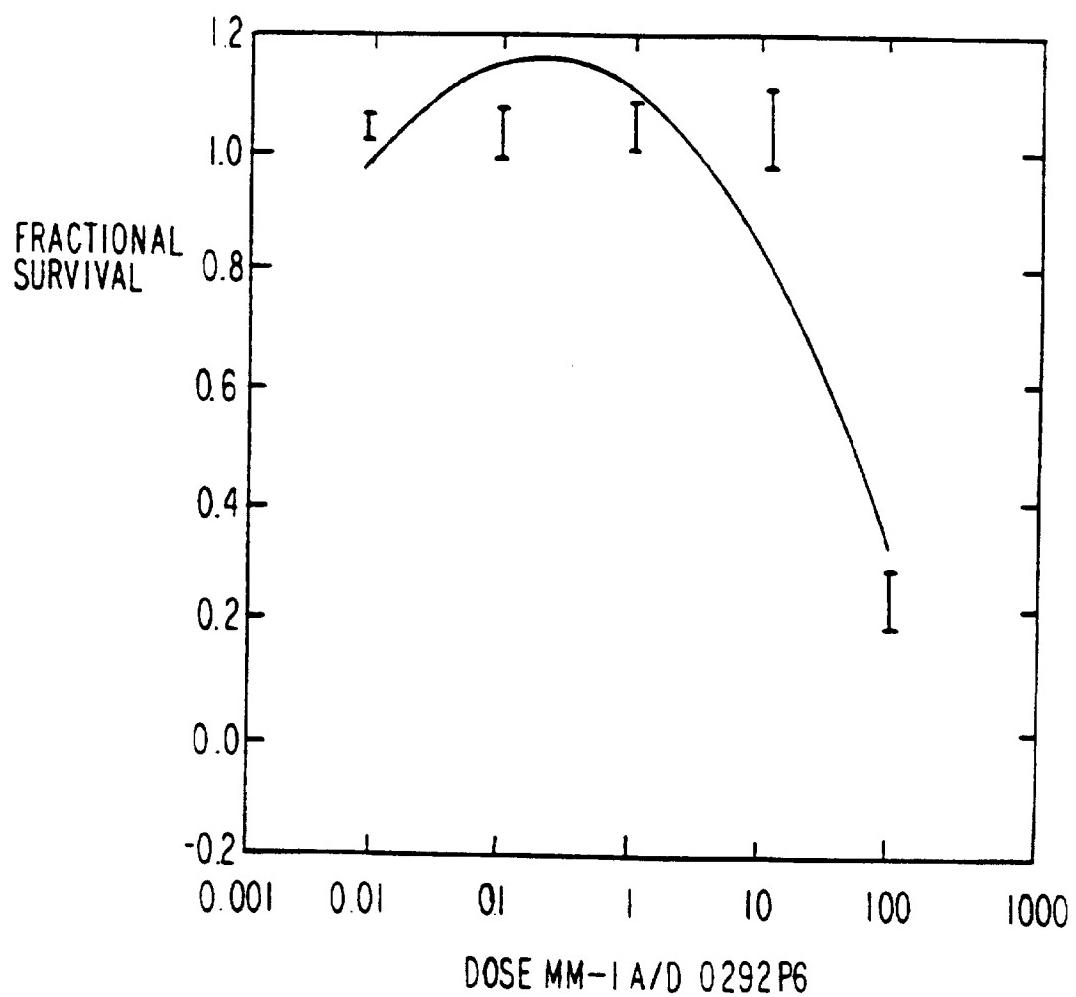
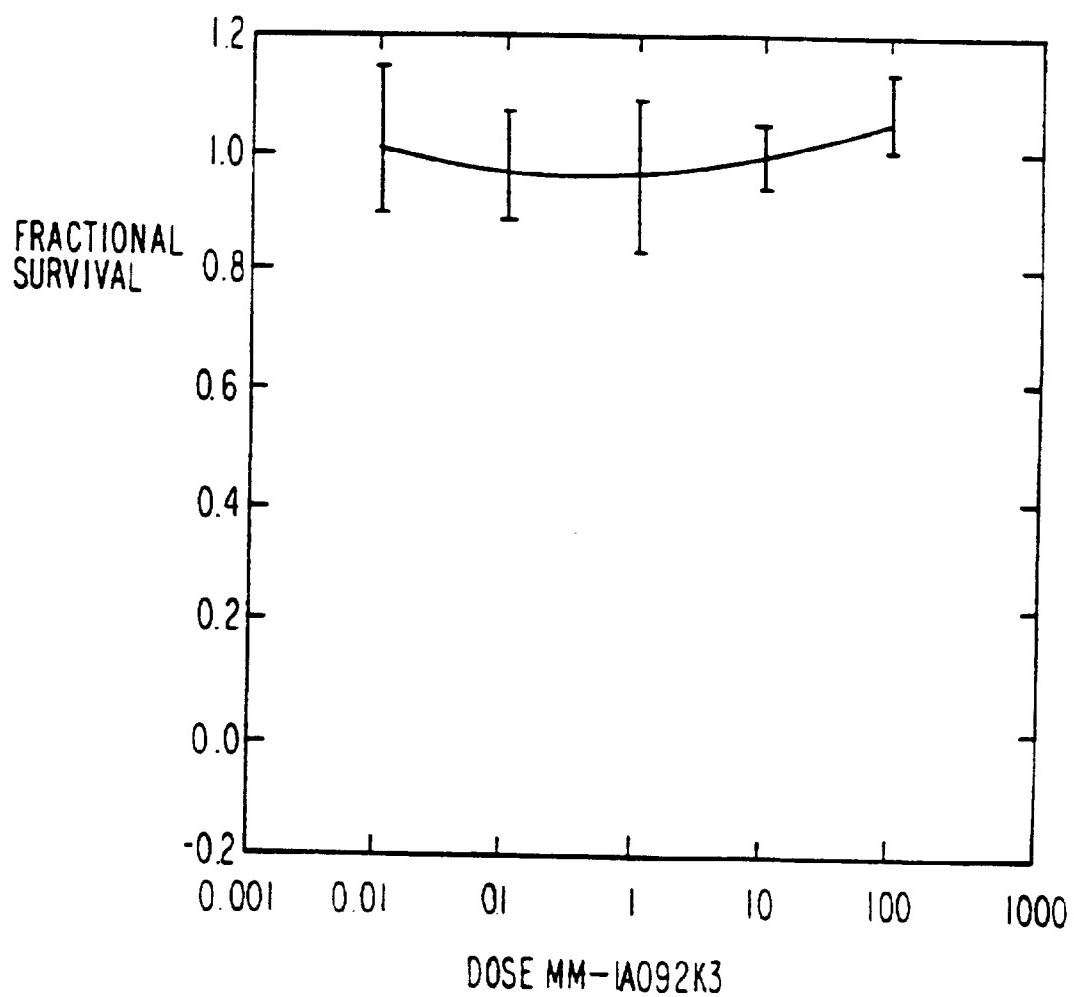
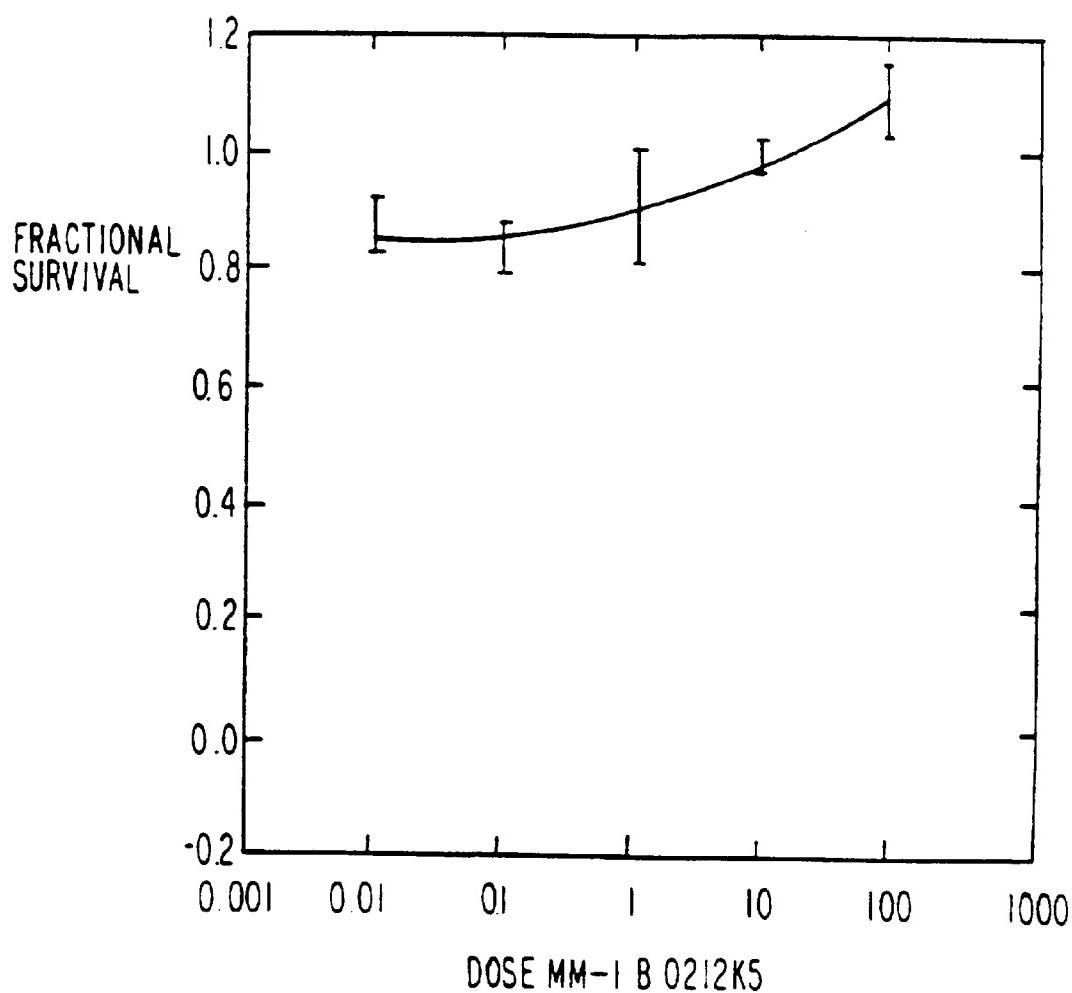


FIG. 7H

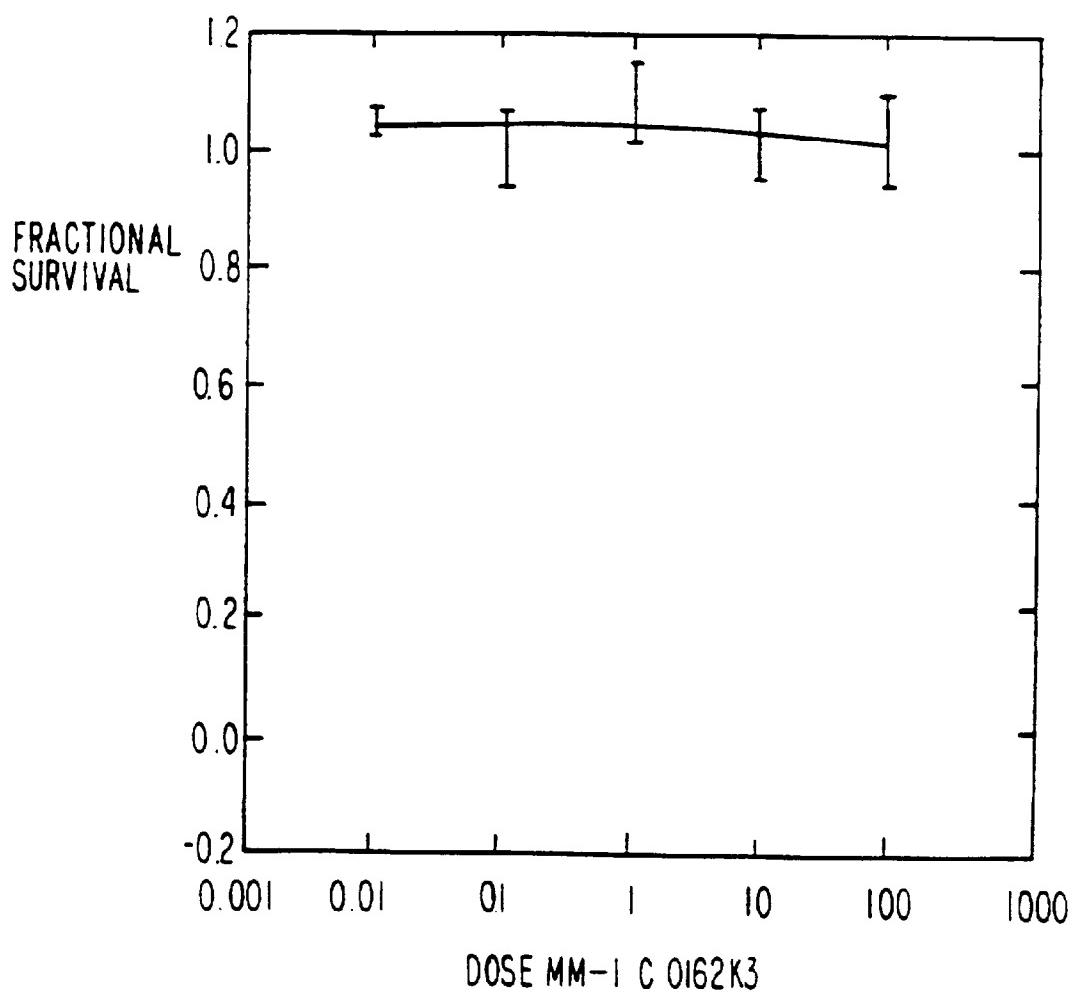
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FIG.8A

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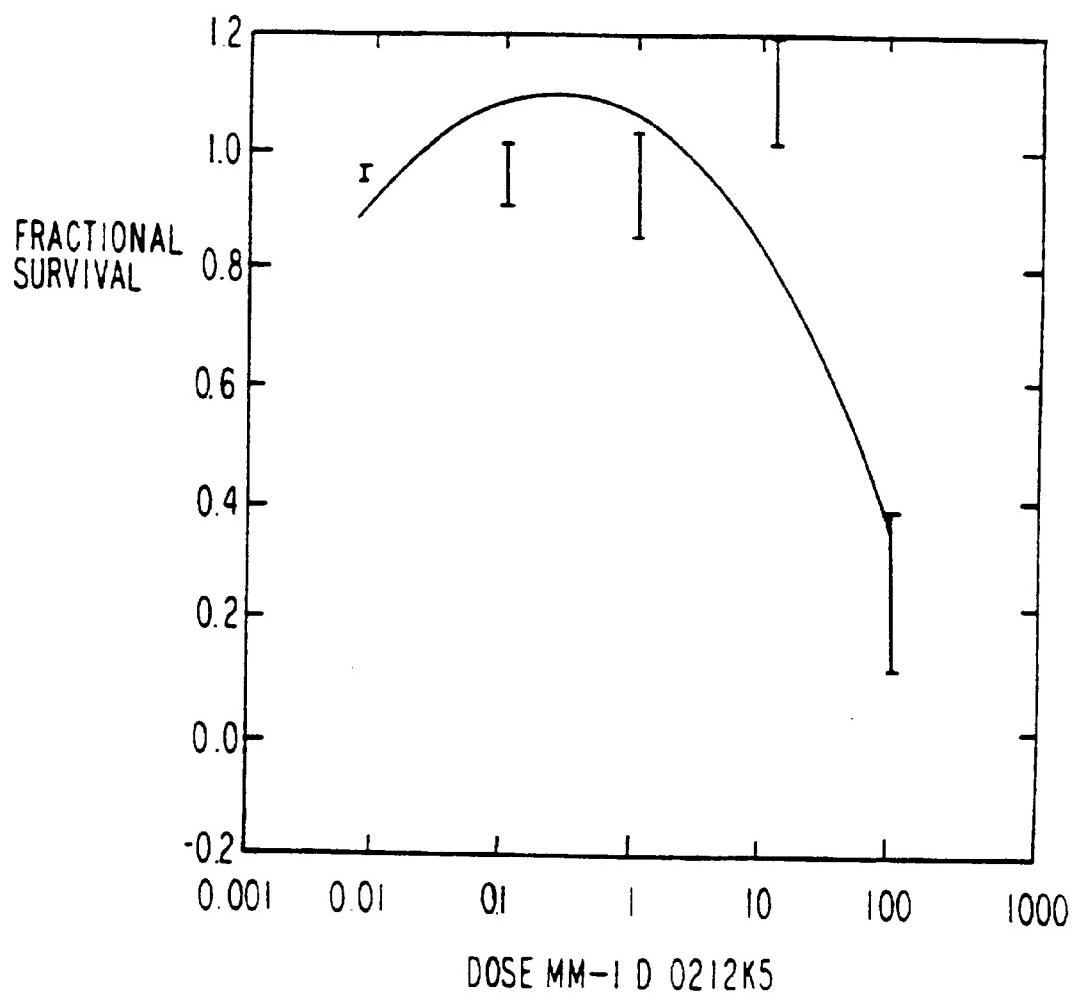
FIG. 8B

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FIG.8C

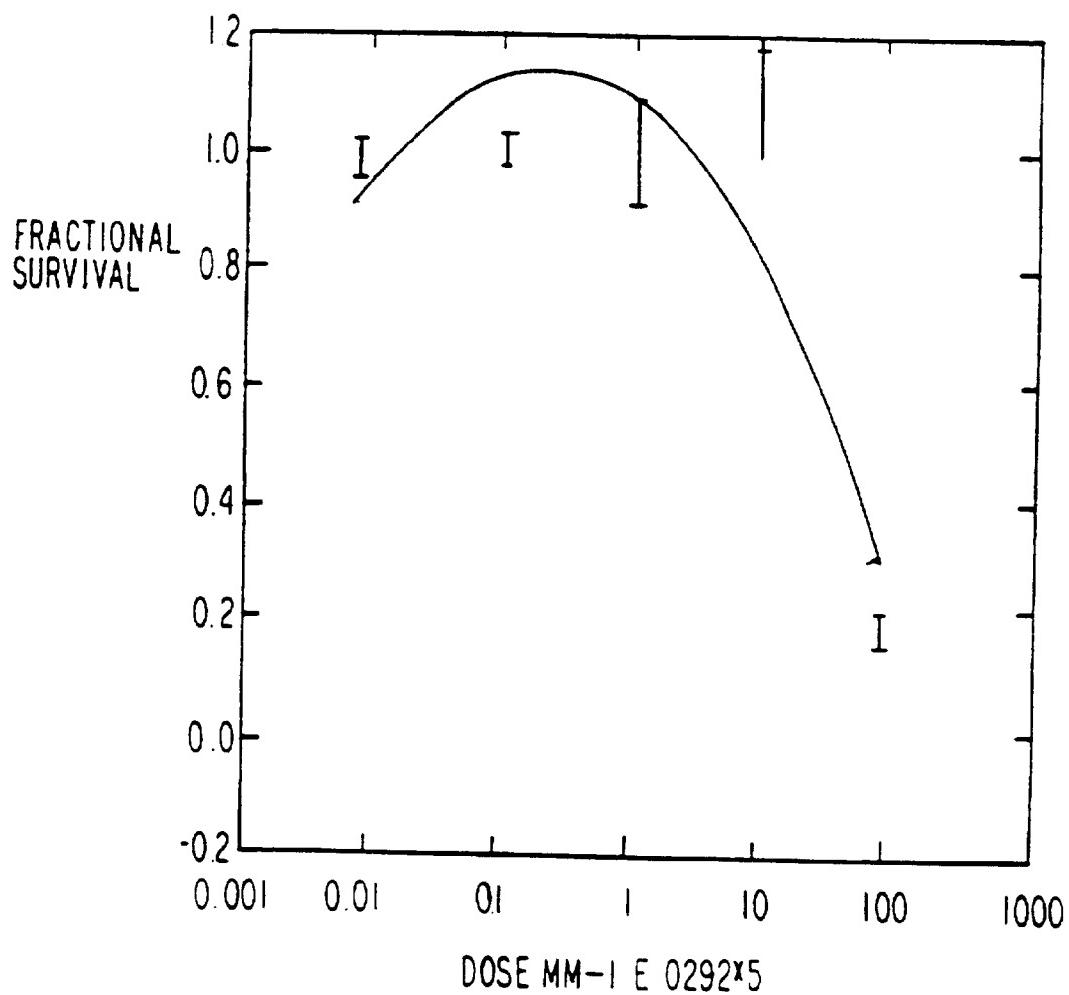
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FIG. 8D



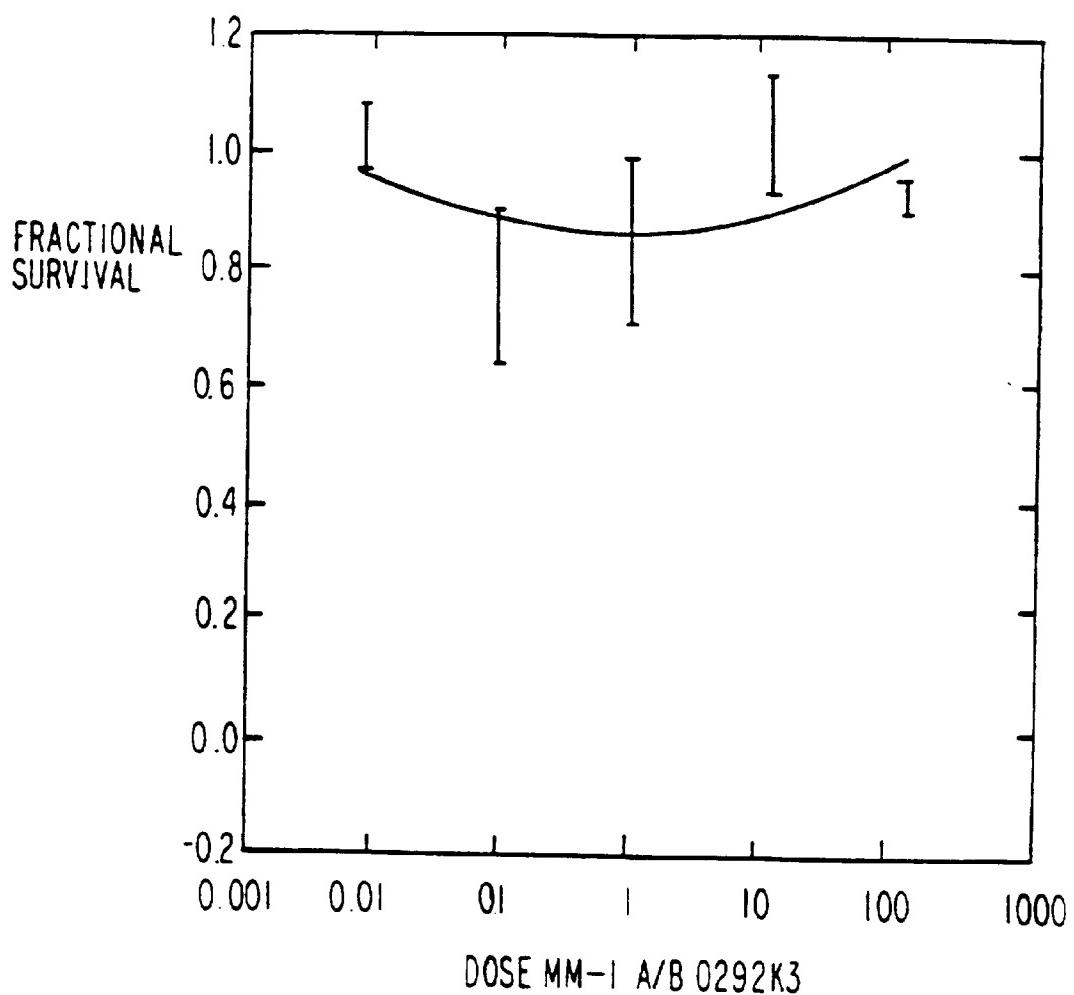
27/235

FIG. 8E

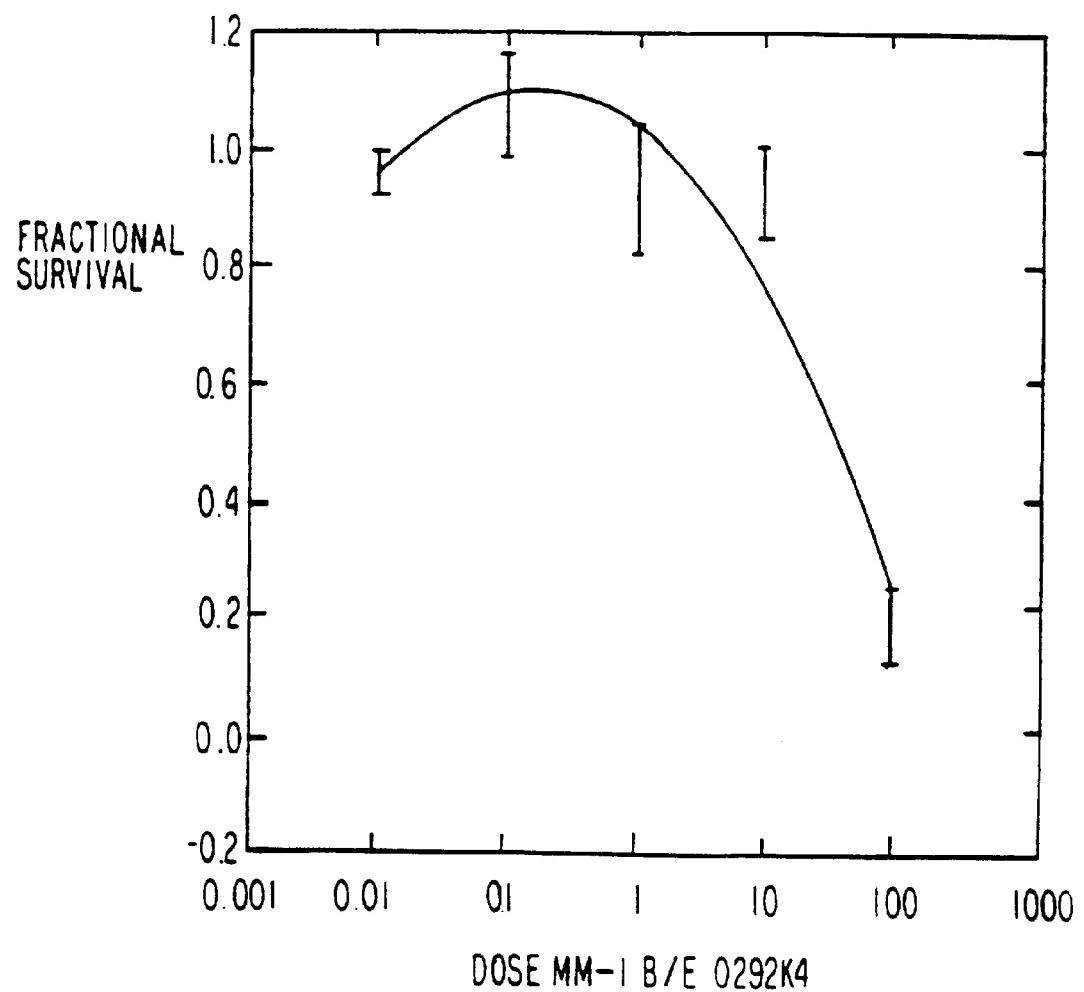


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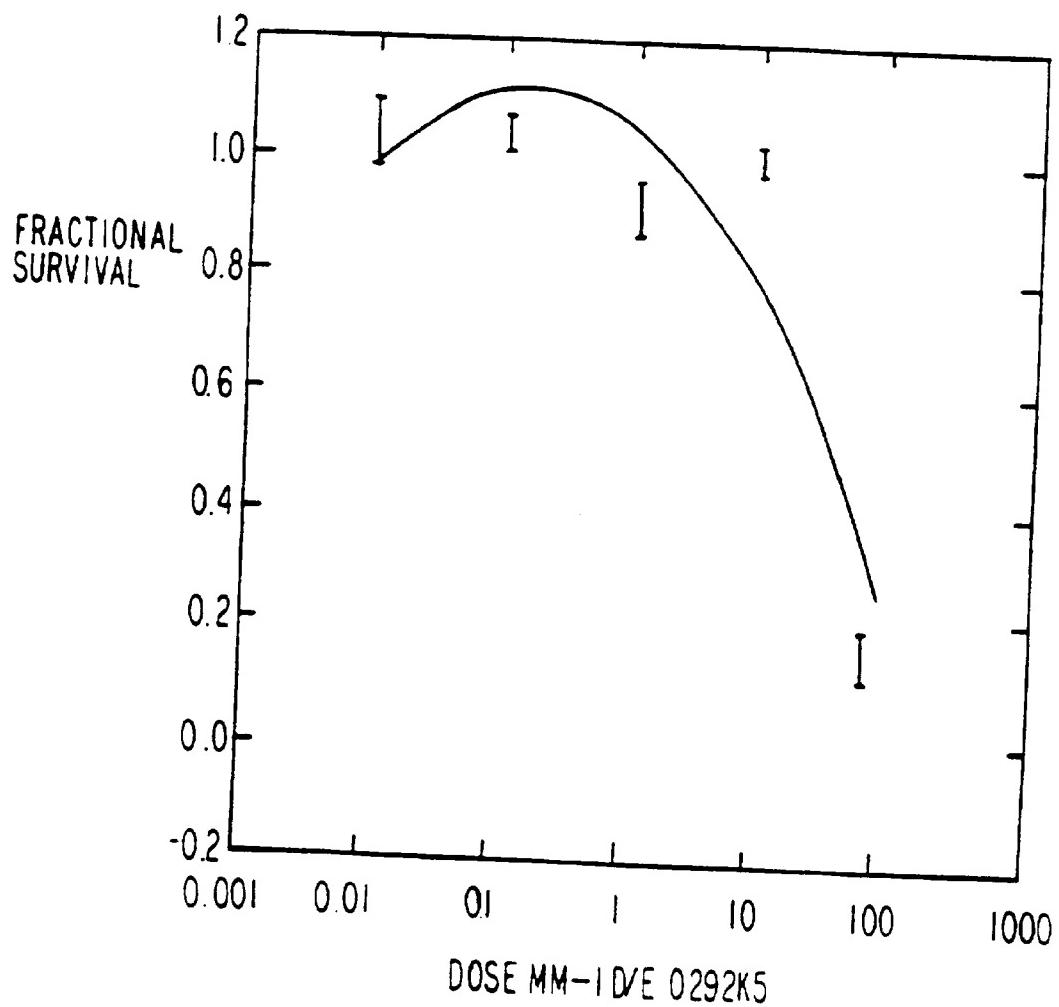
FIG. 8F



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FIG. 8G

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FIG. 8H

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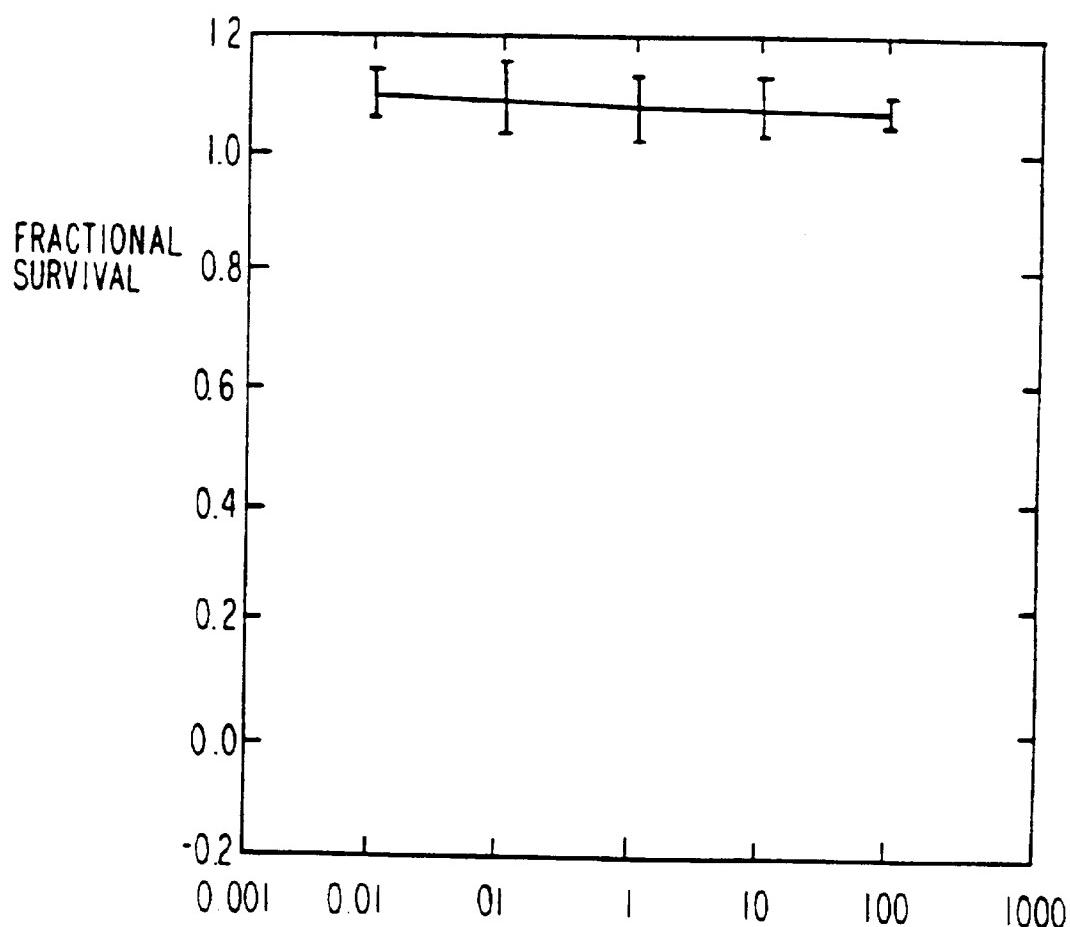
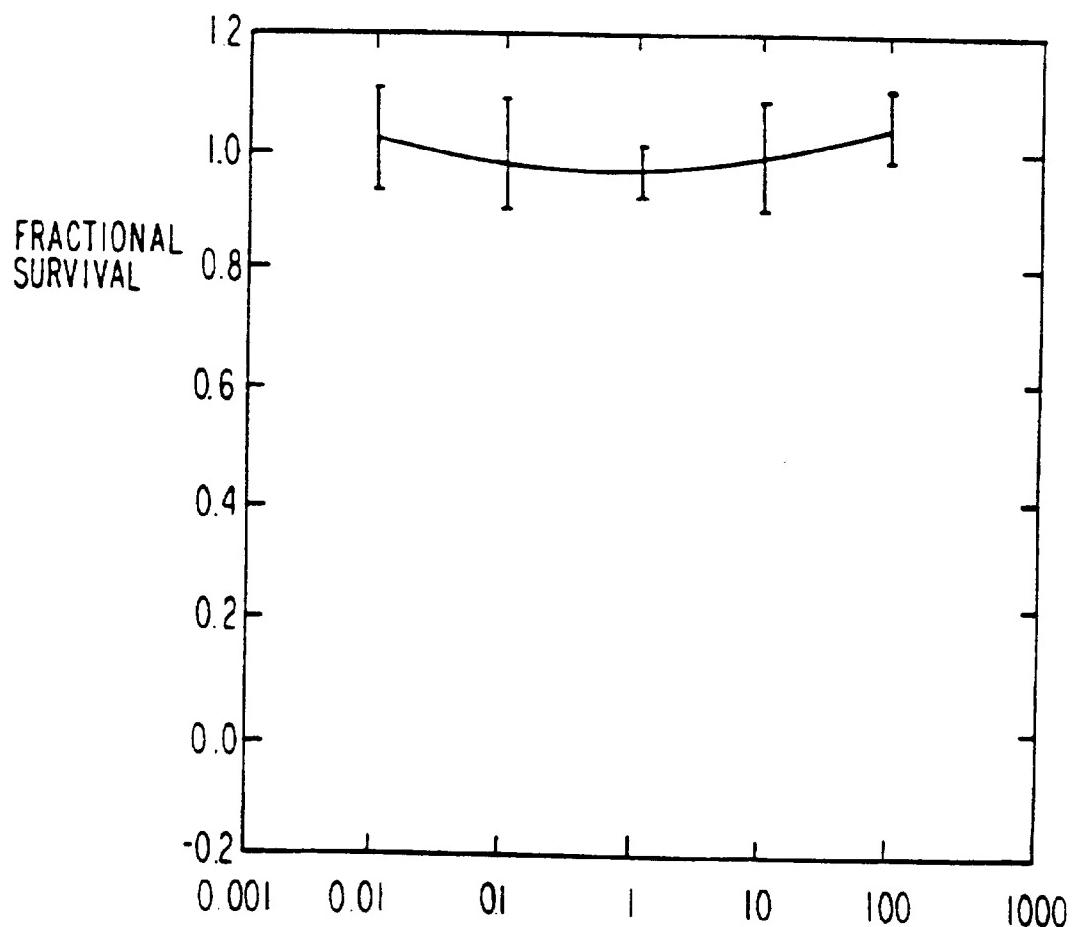
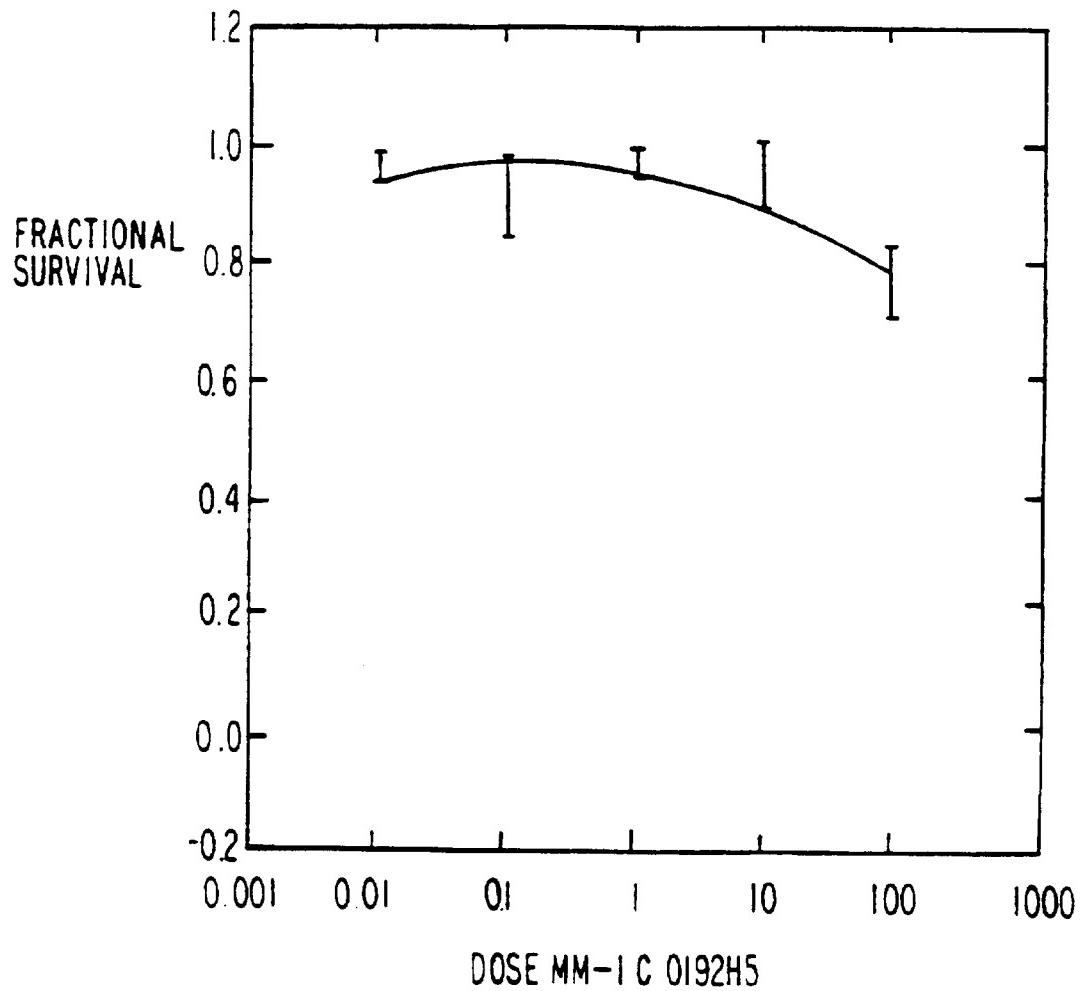
FIG. 9A

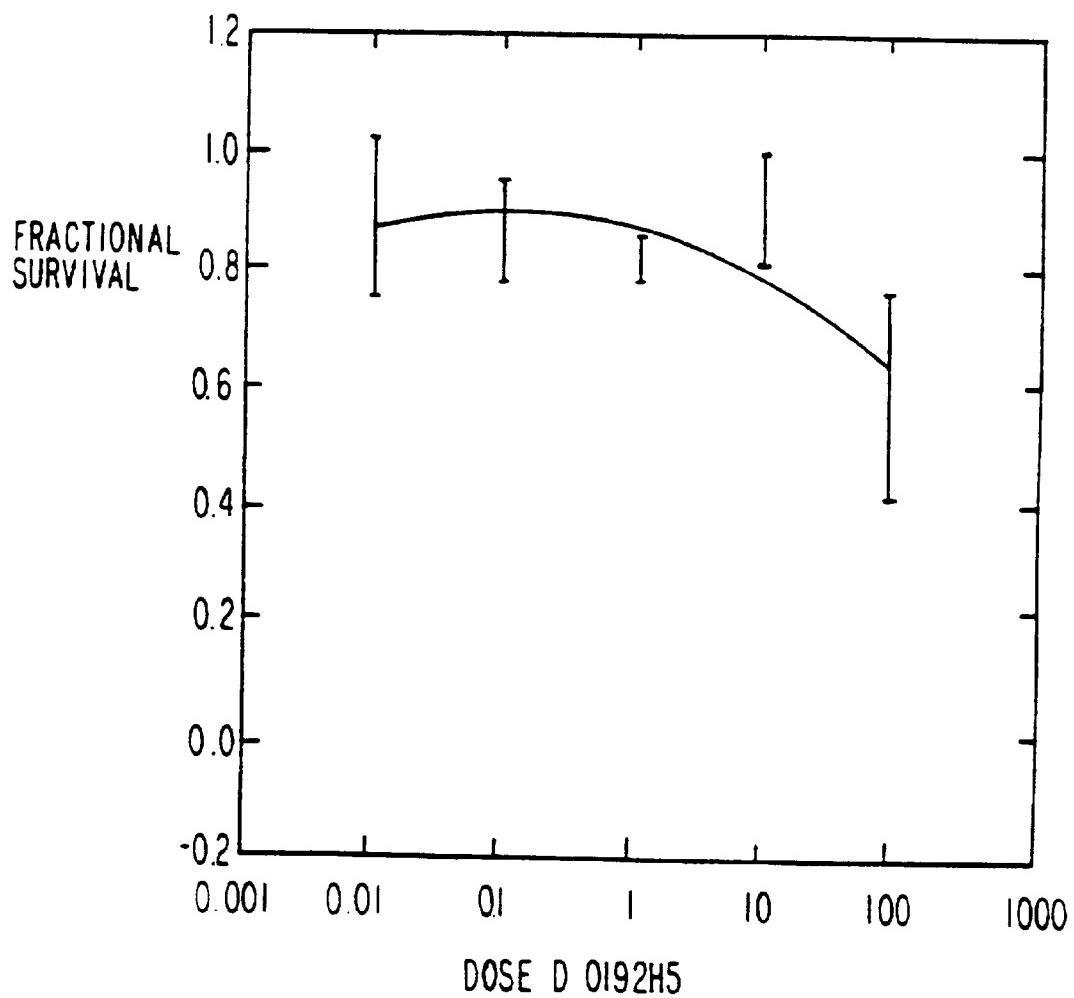
FIG. 9B

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FIG. 9C

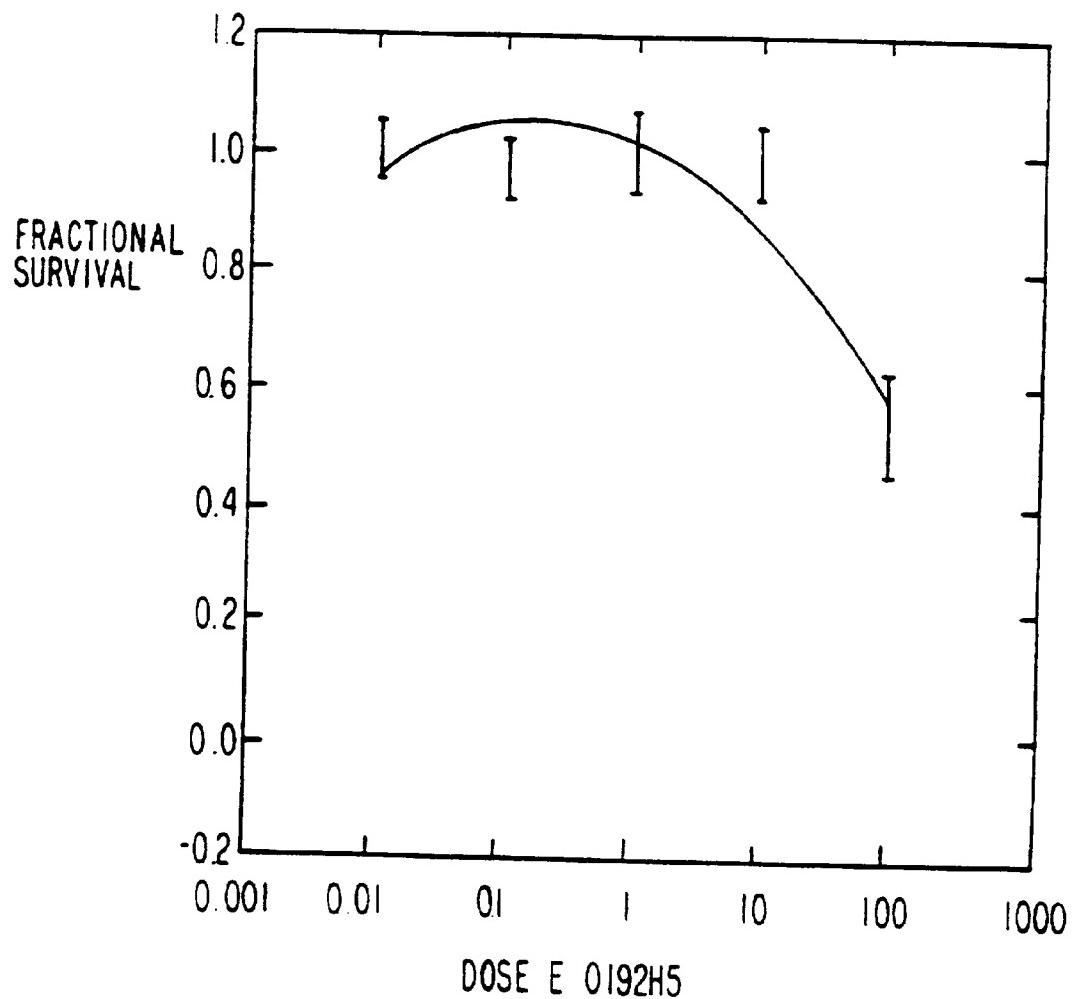
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FIG. 9D



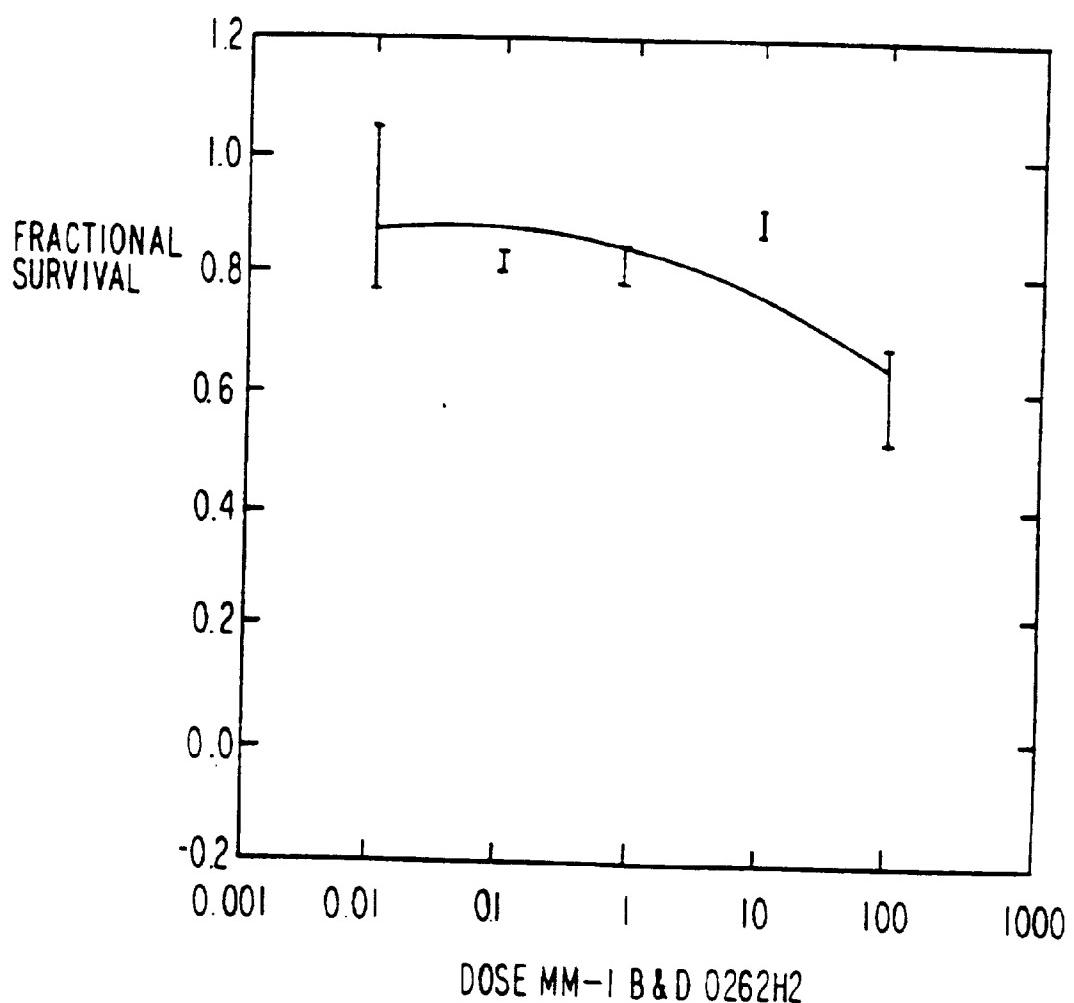
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FIG. 9E



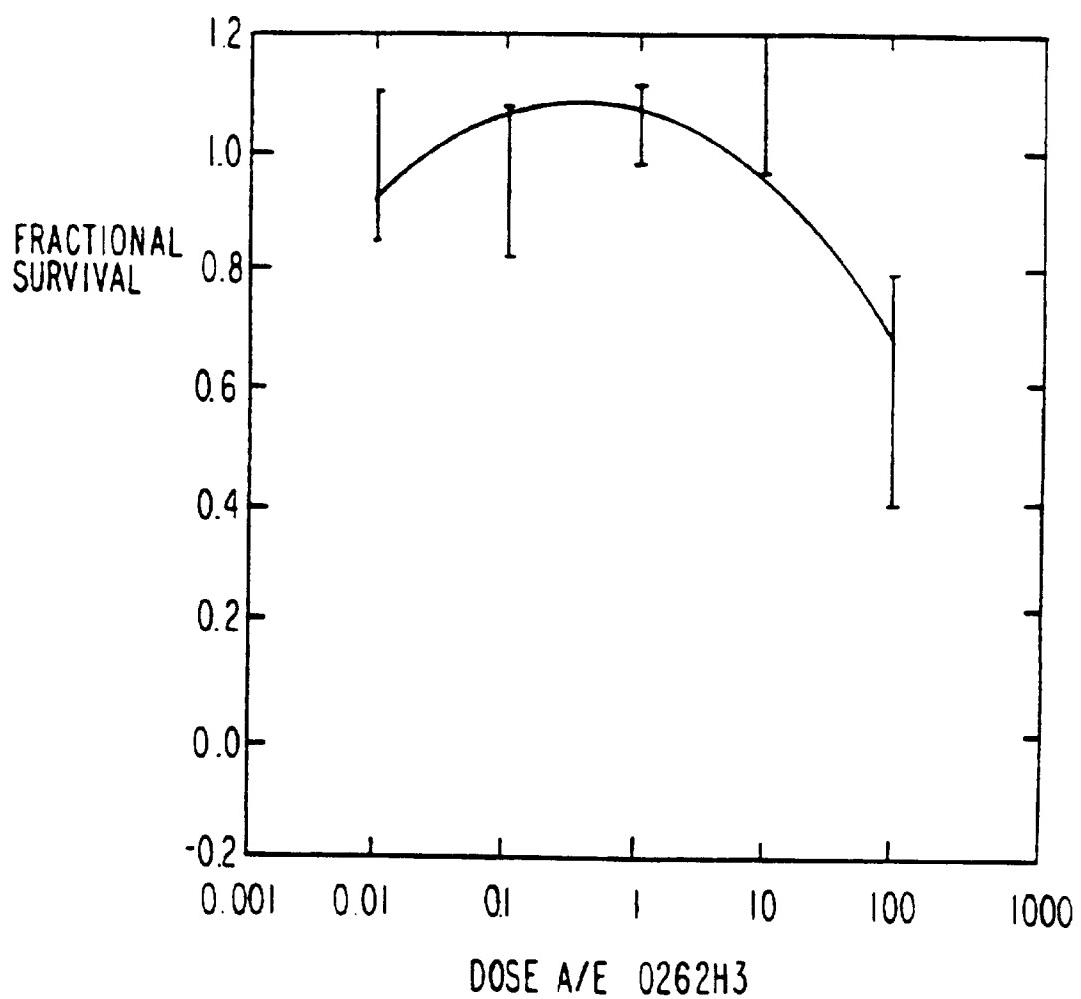
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FIG. 9F



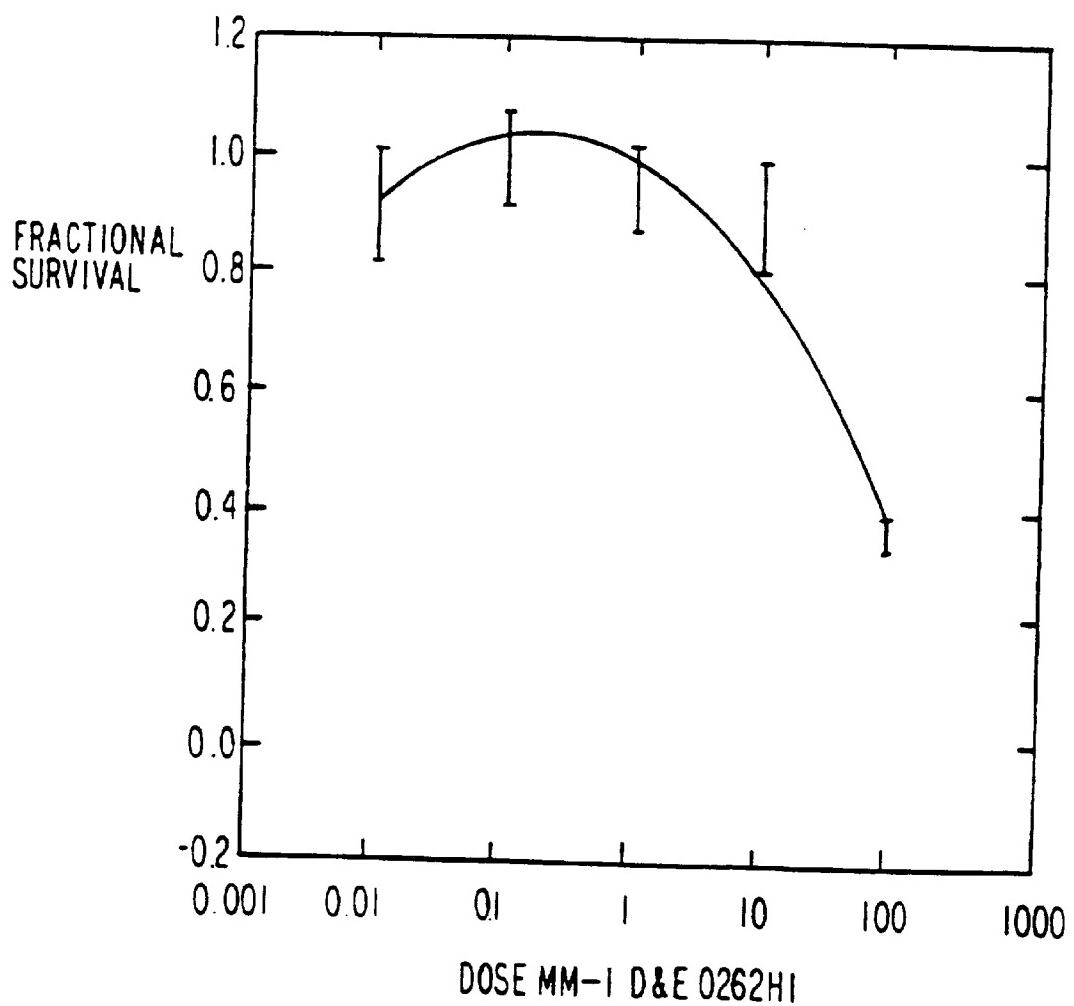
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FIG.9G



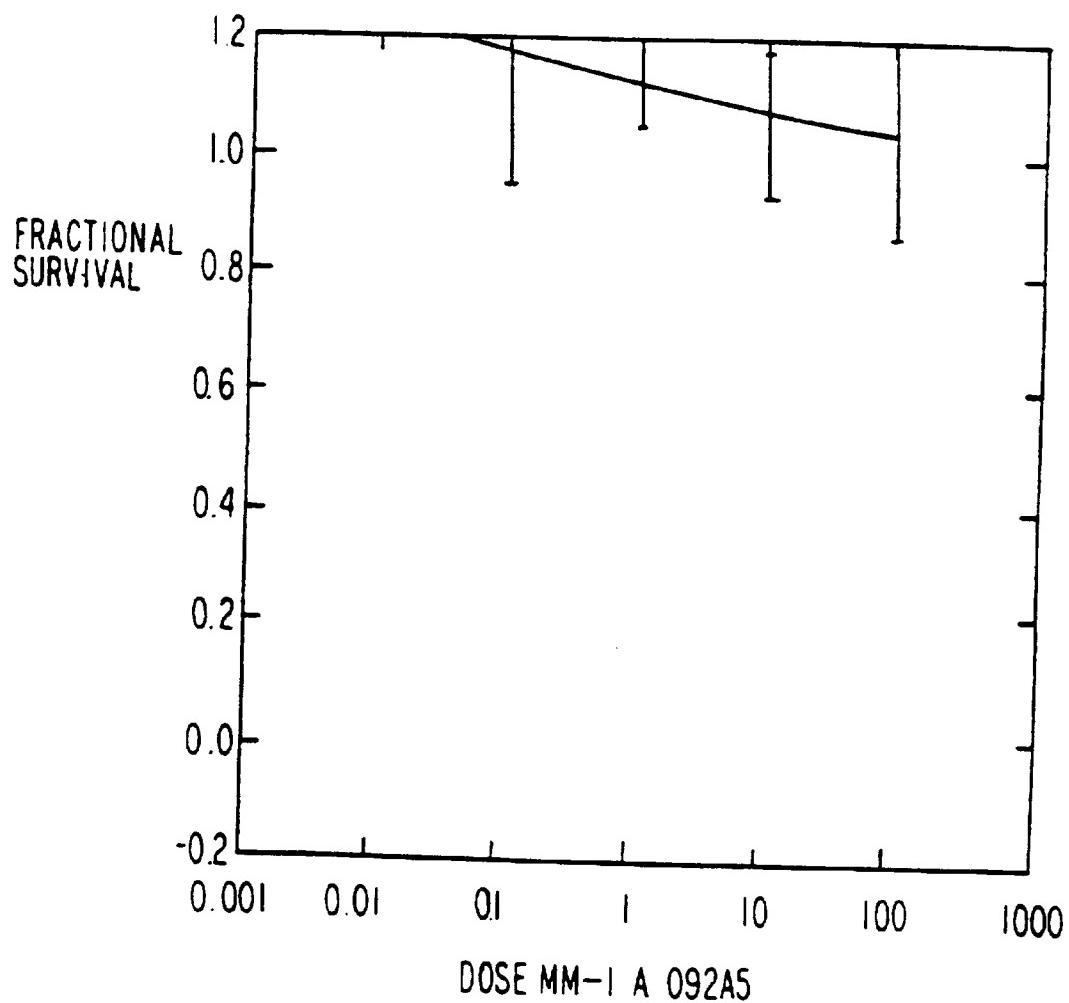
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FIG. 9H

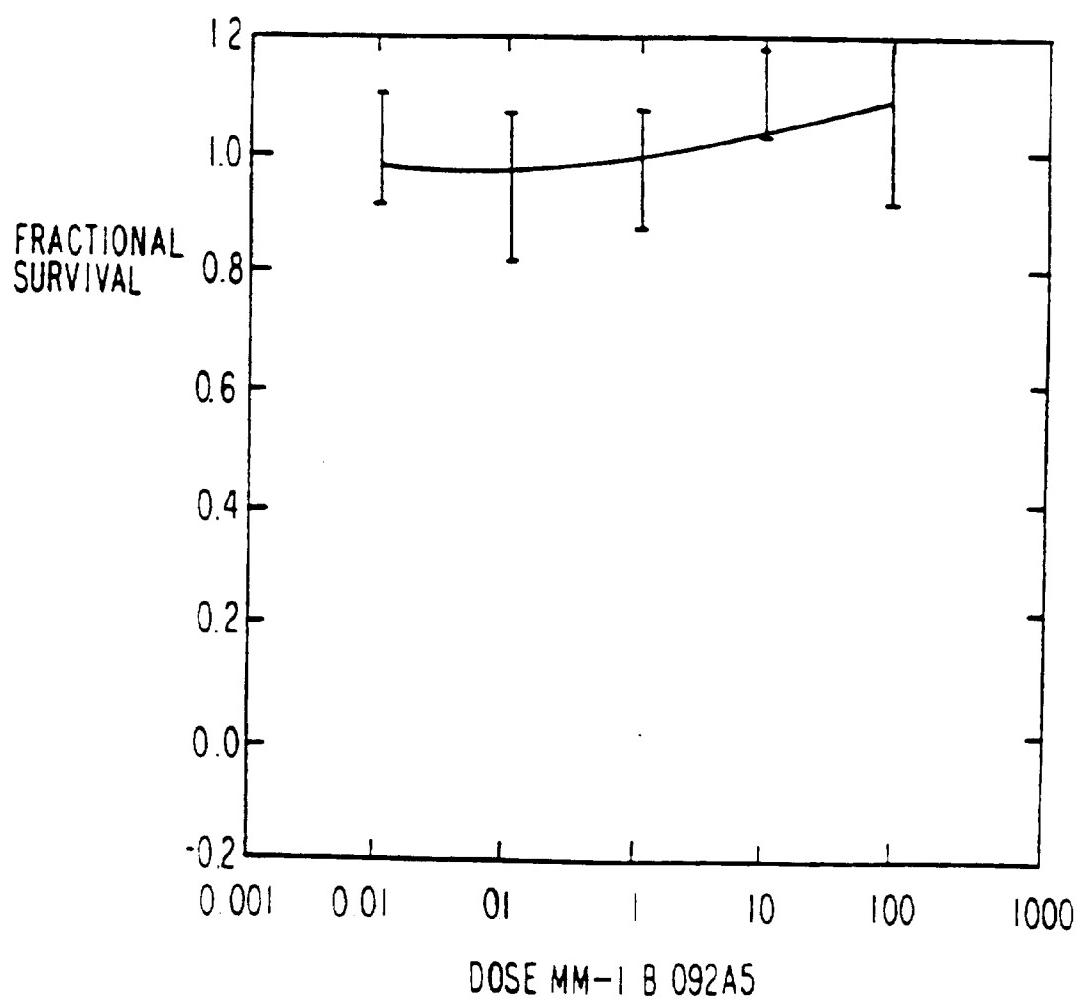


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FIG. 10A

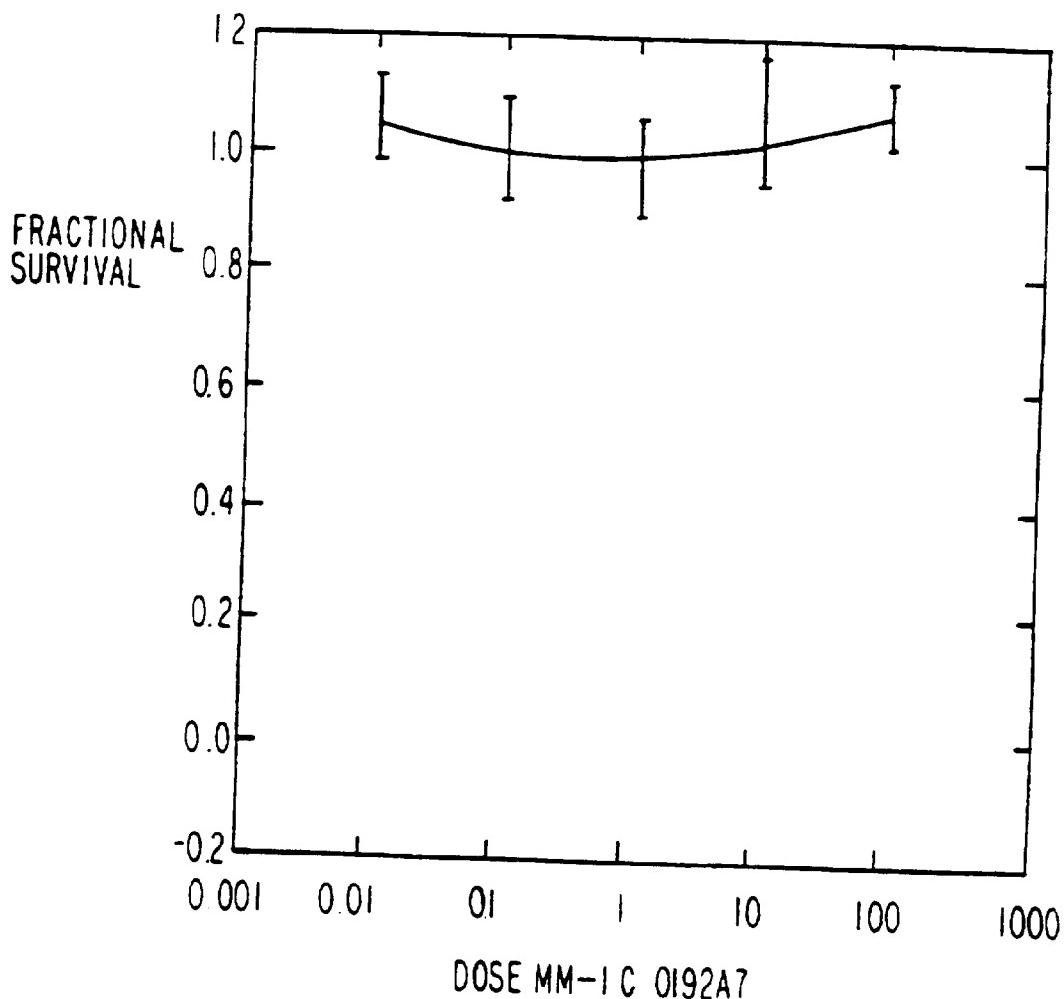


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FIG. 10B

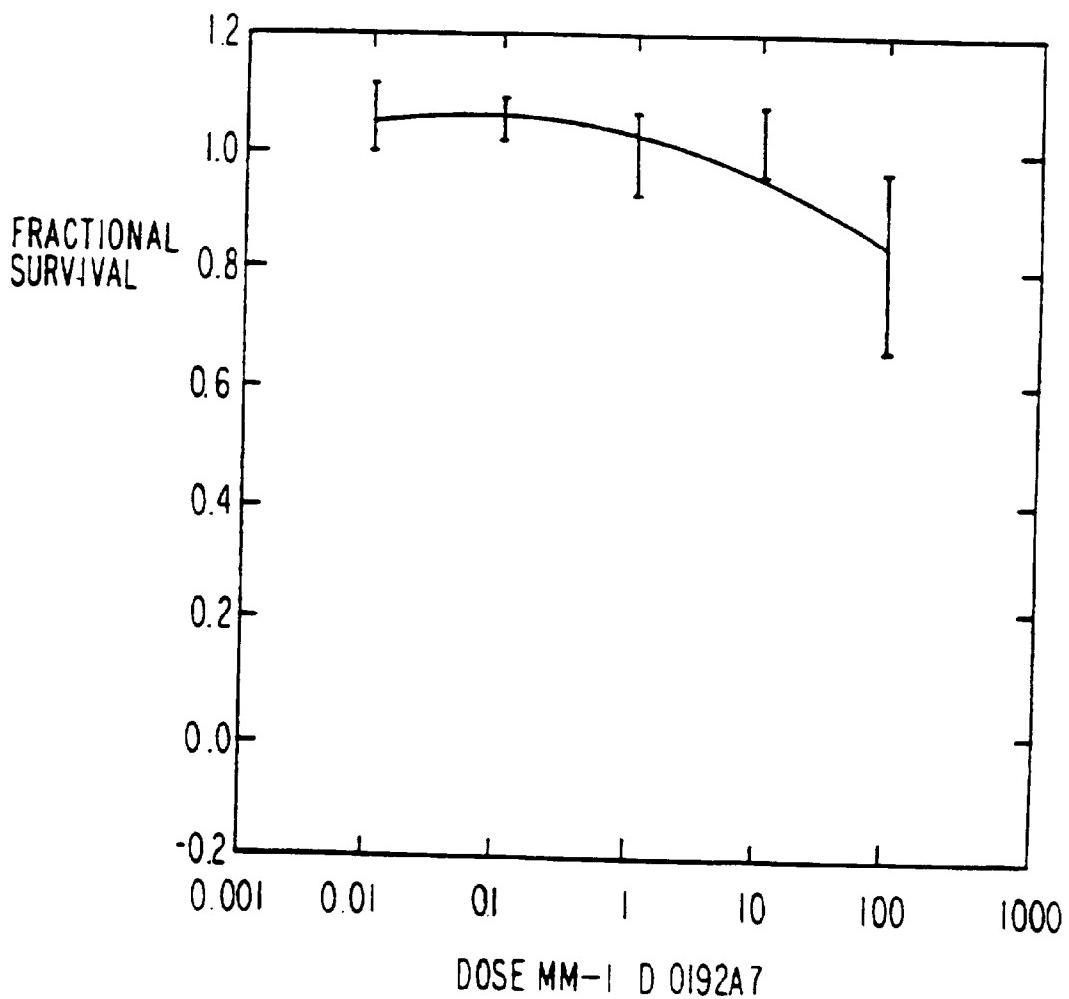
41/235

FIG. 10C



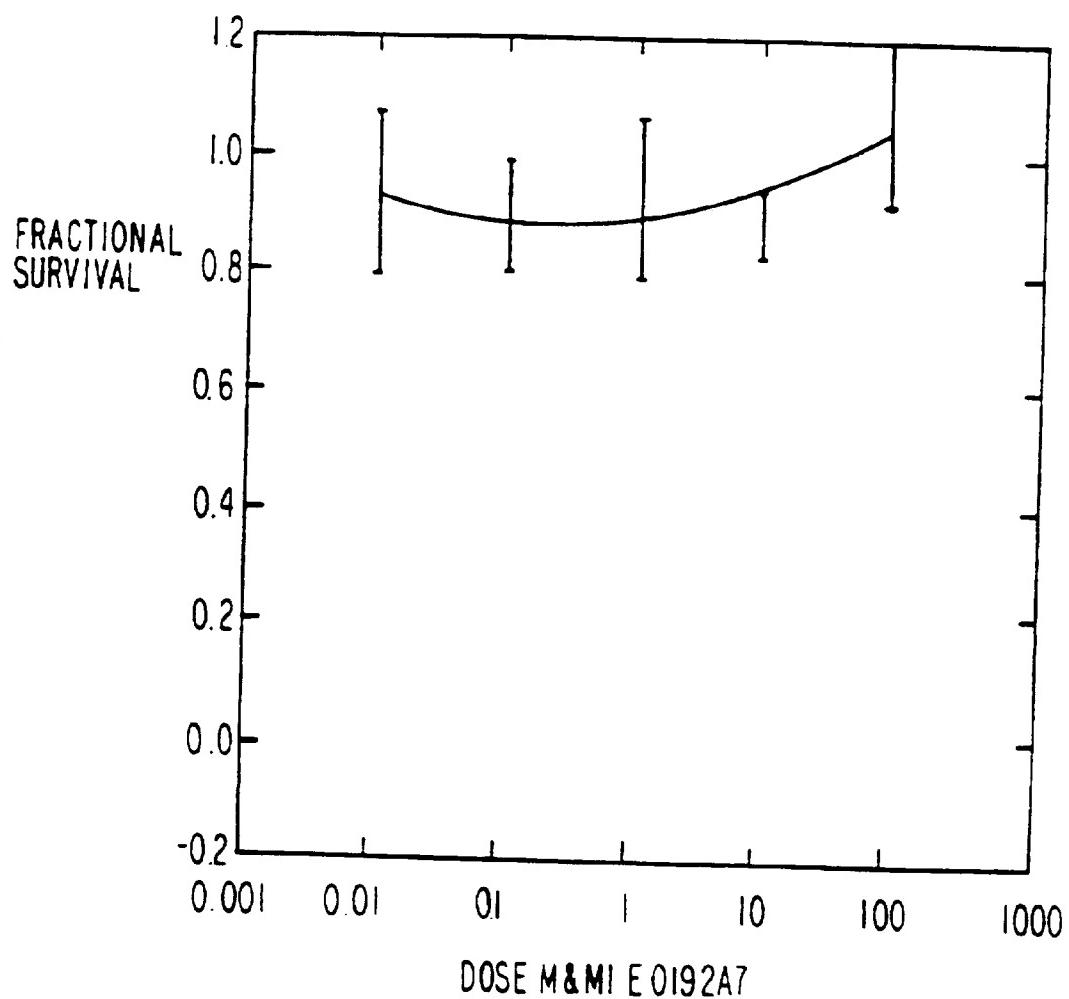
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FIG. 10D



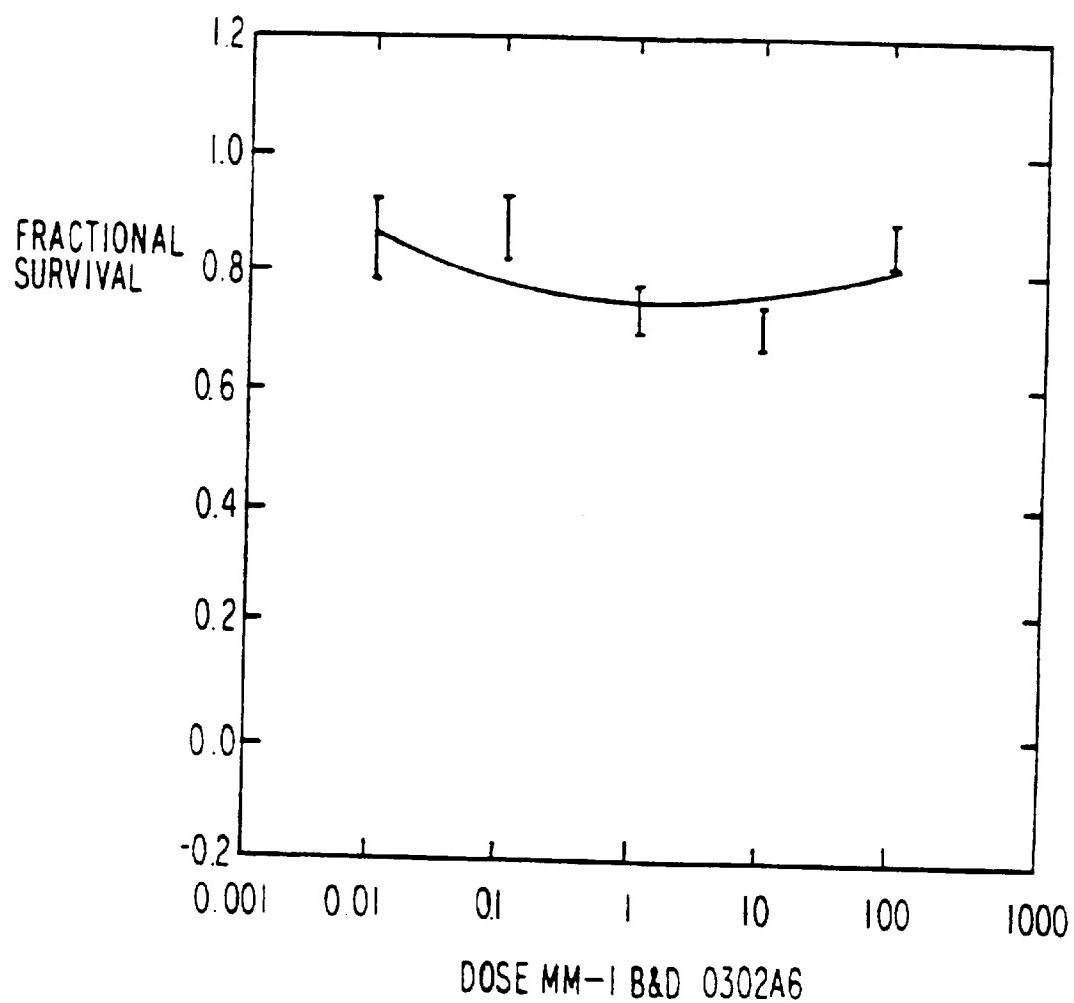
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FIG. 10E



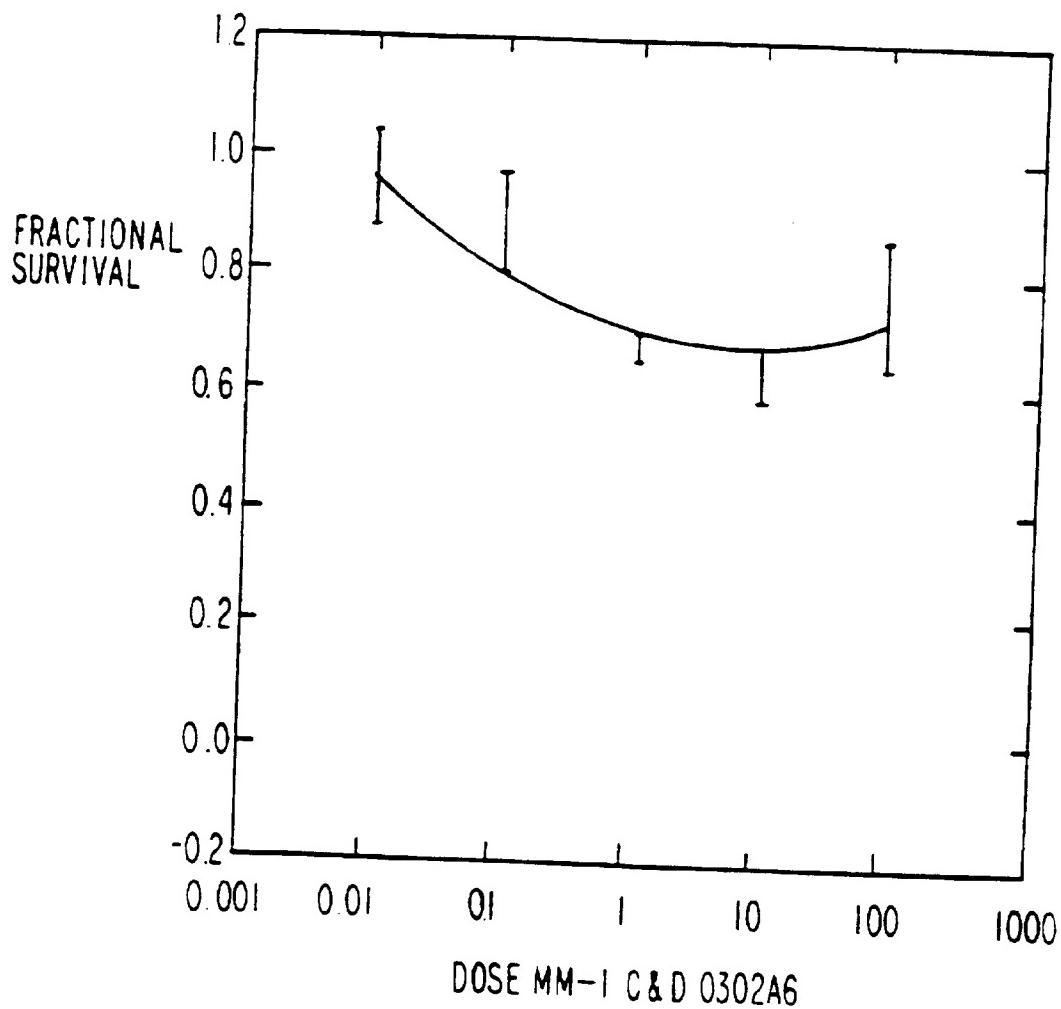
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FIG. 10F



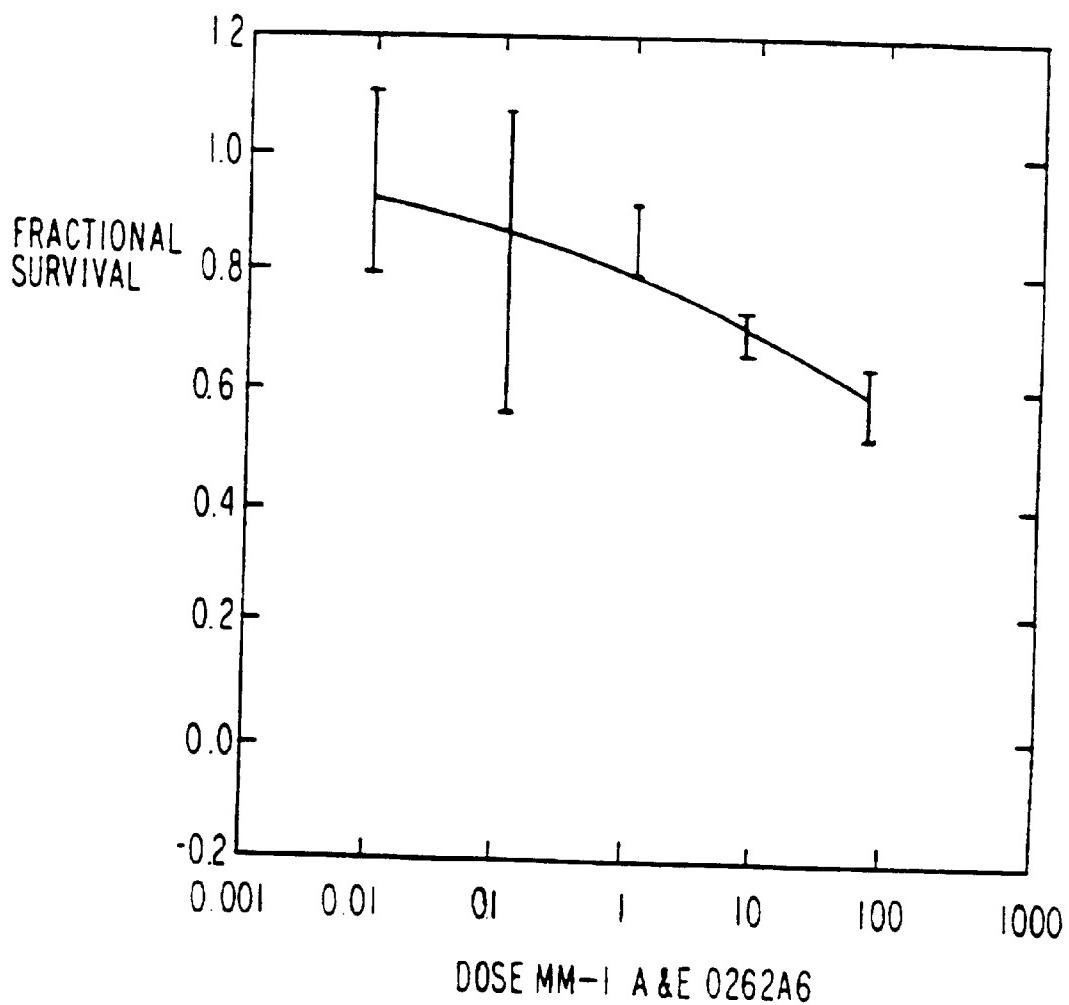
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FIG.10G



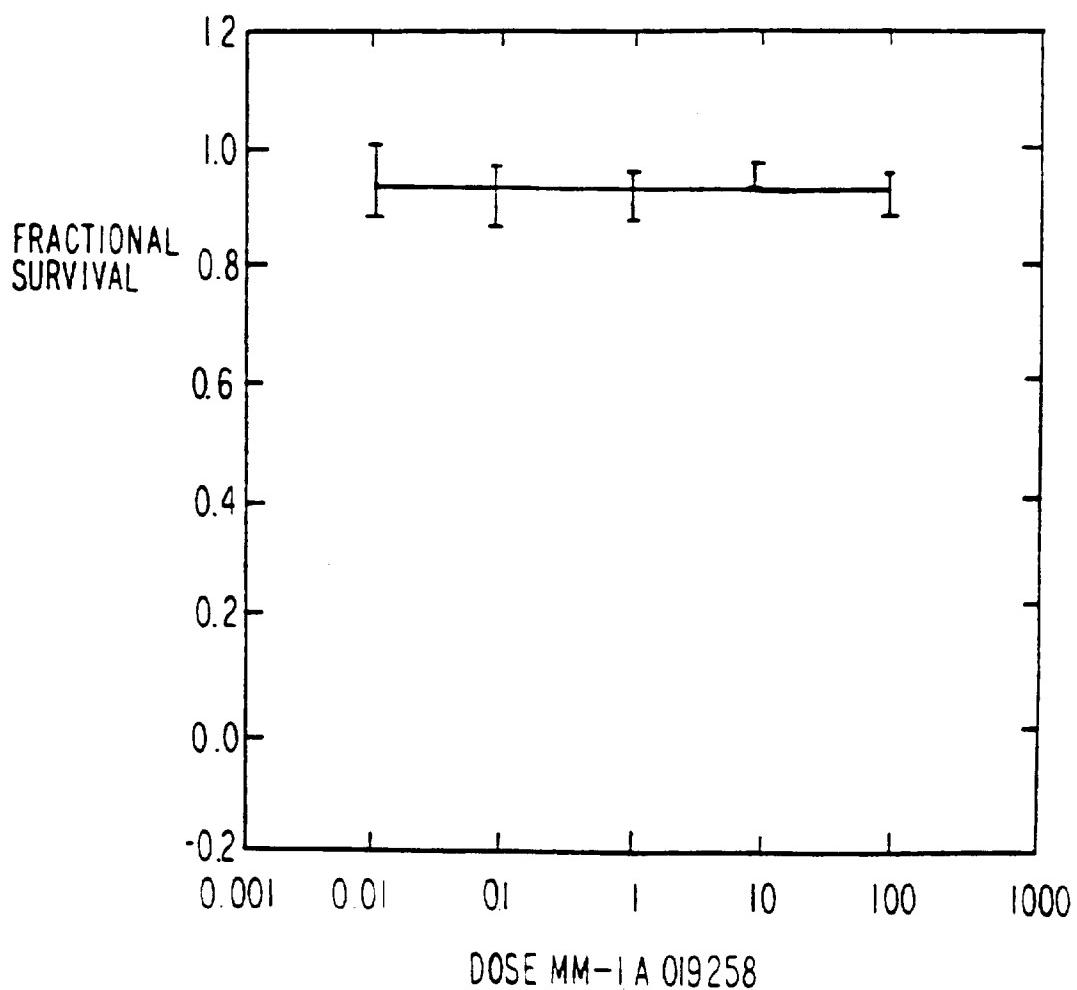
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FIG. 10H



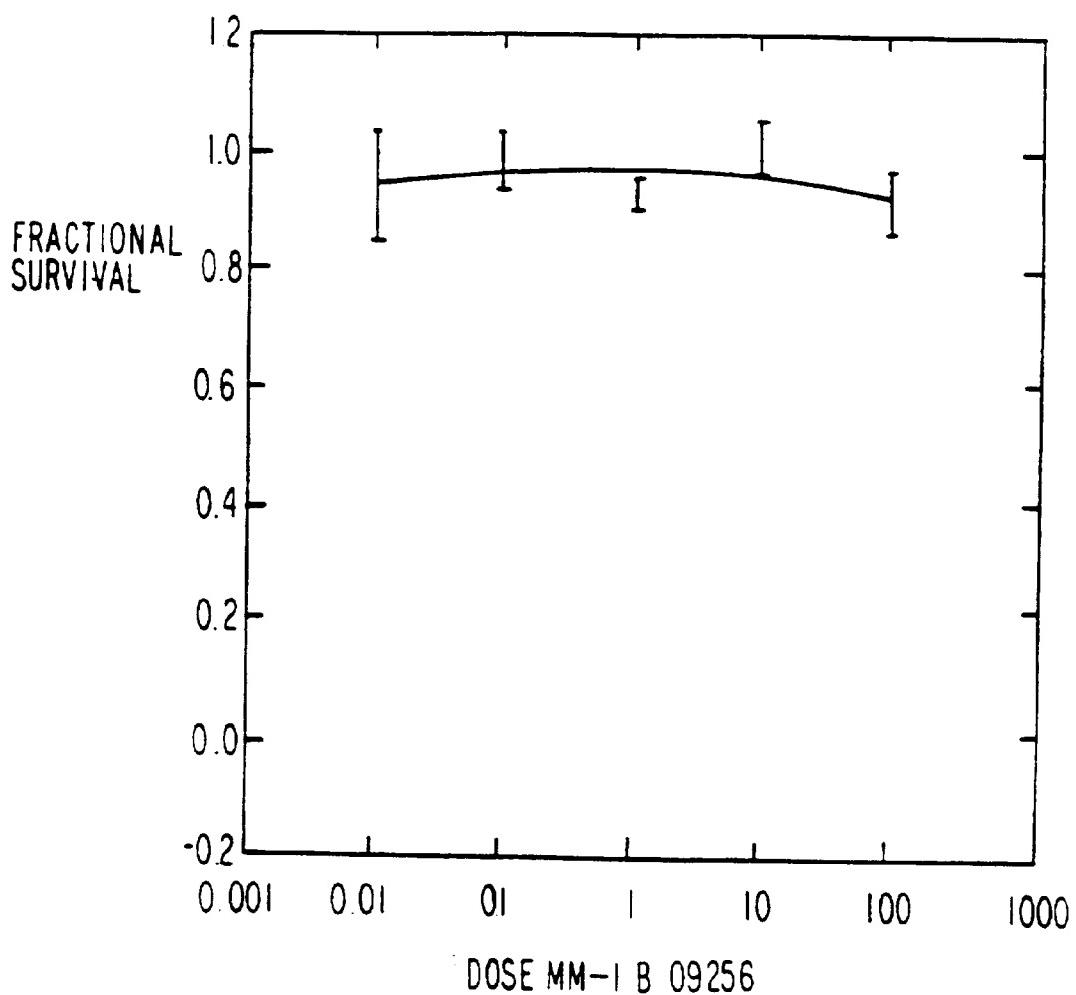
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FIG. 11A



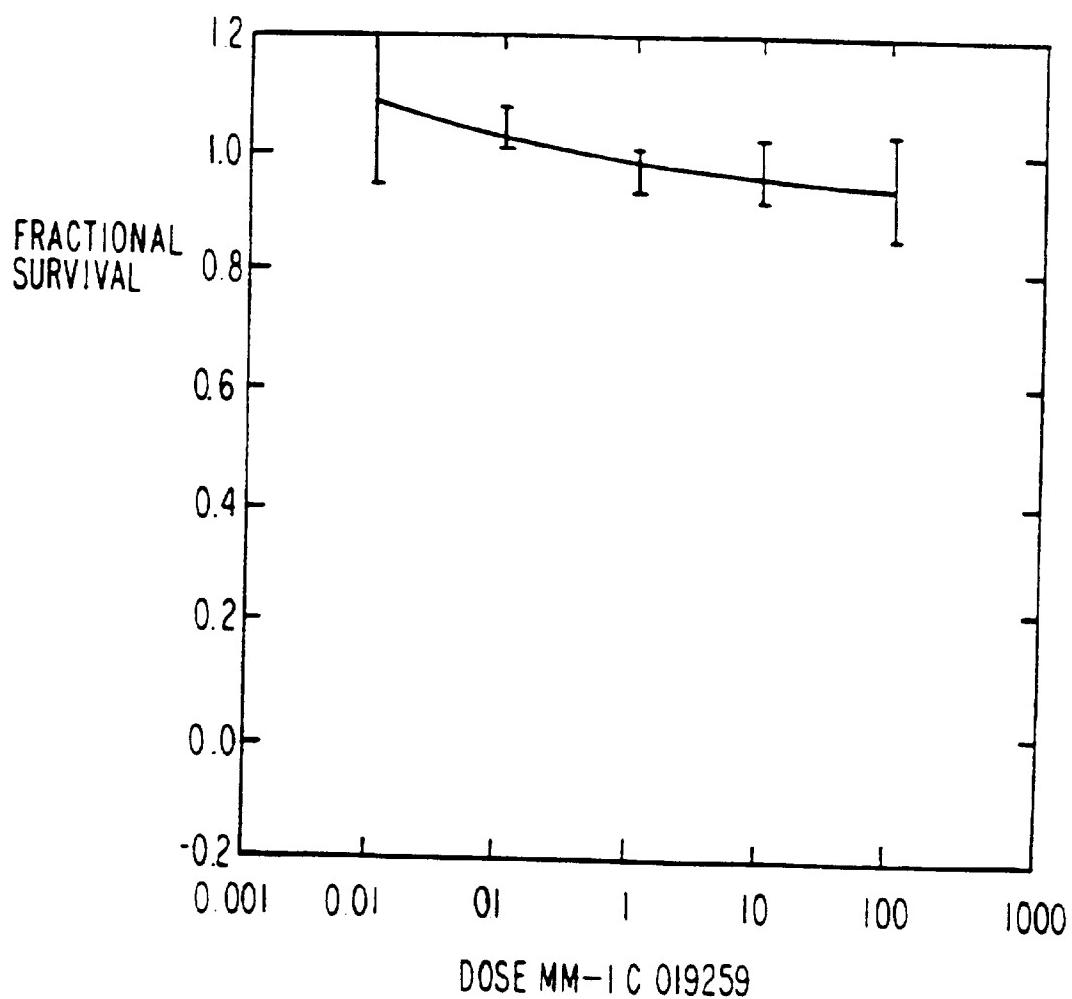
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FIG. 11B



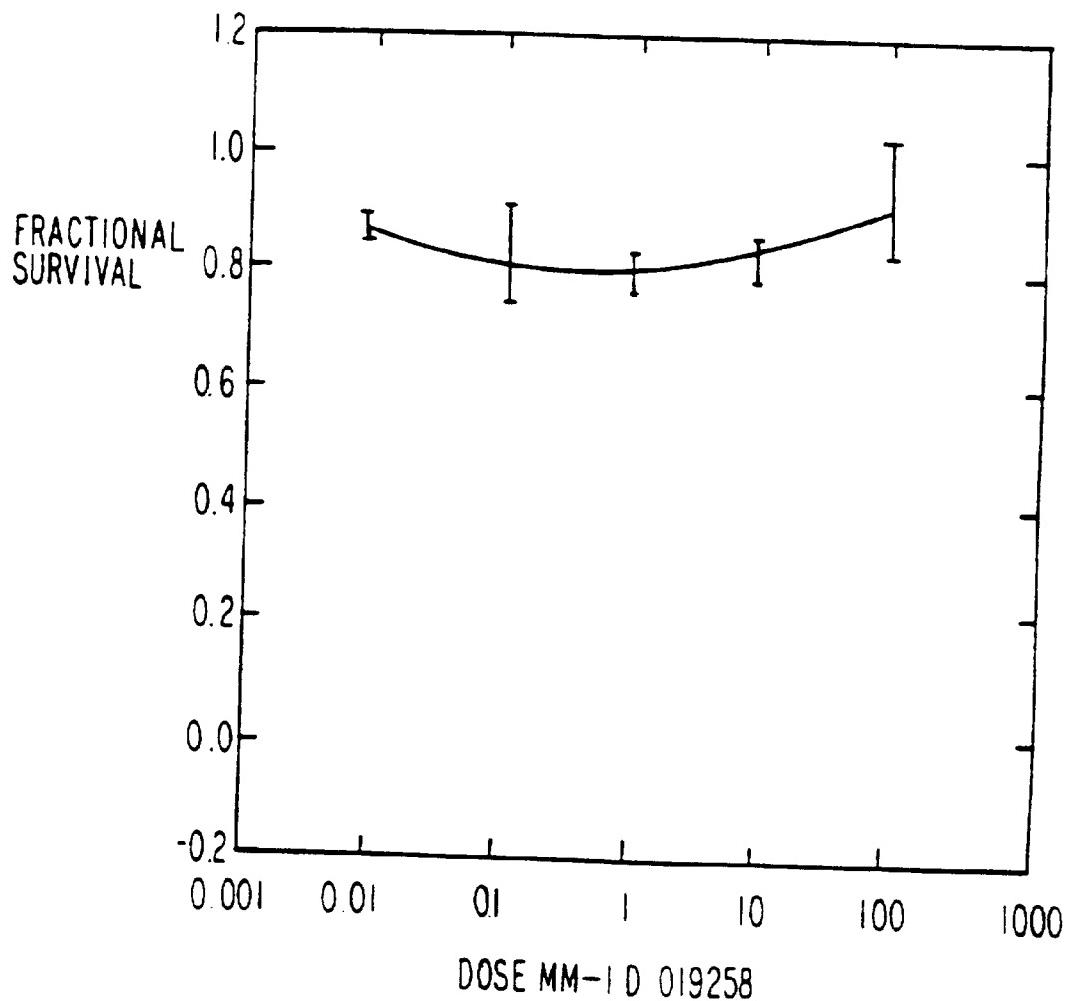
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FIG. 11C



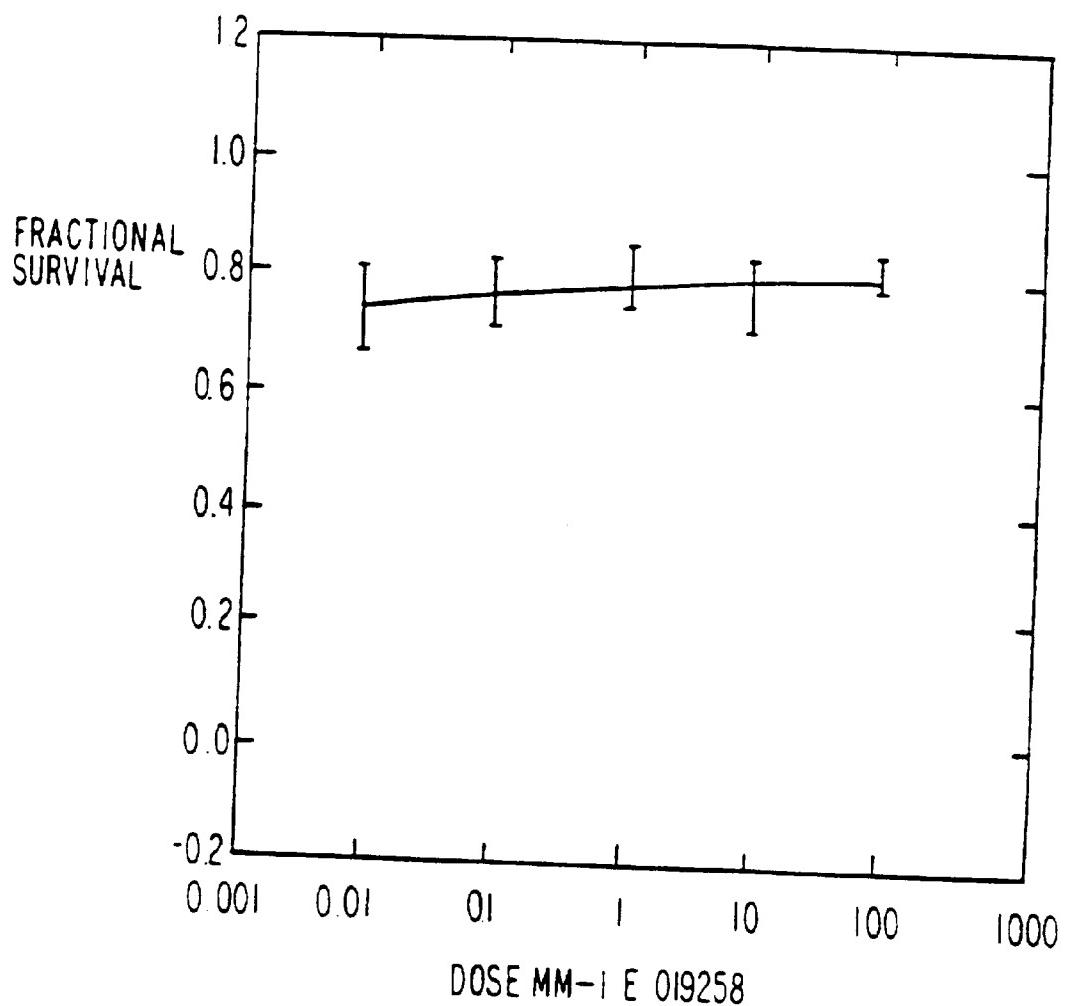
50/235

FIG. 11D



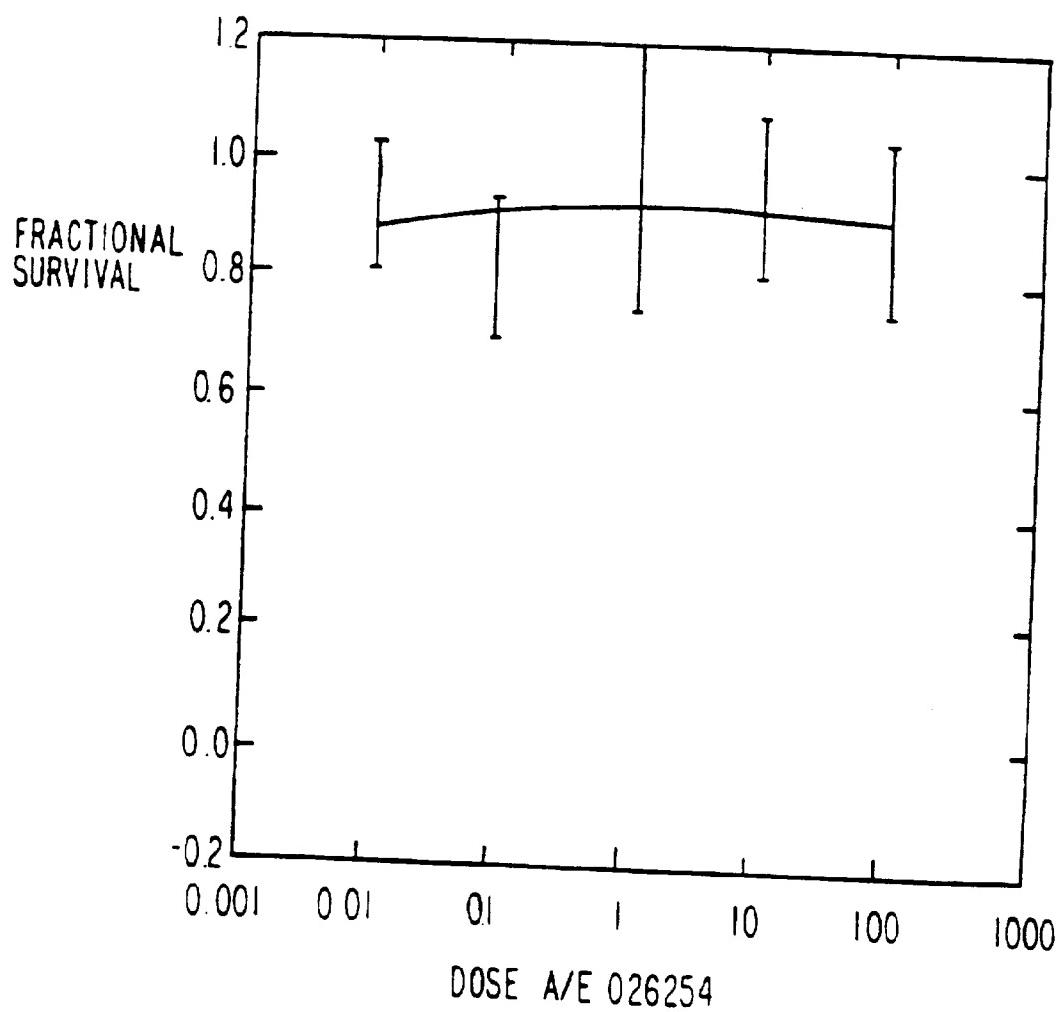
51/235

FIG. 11E

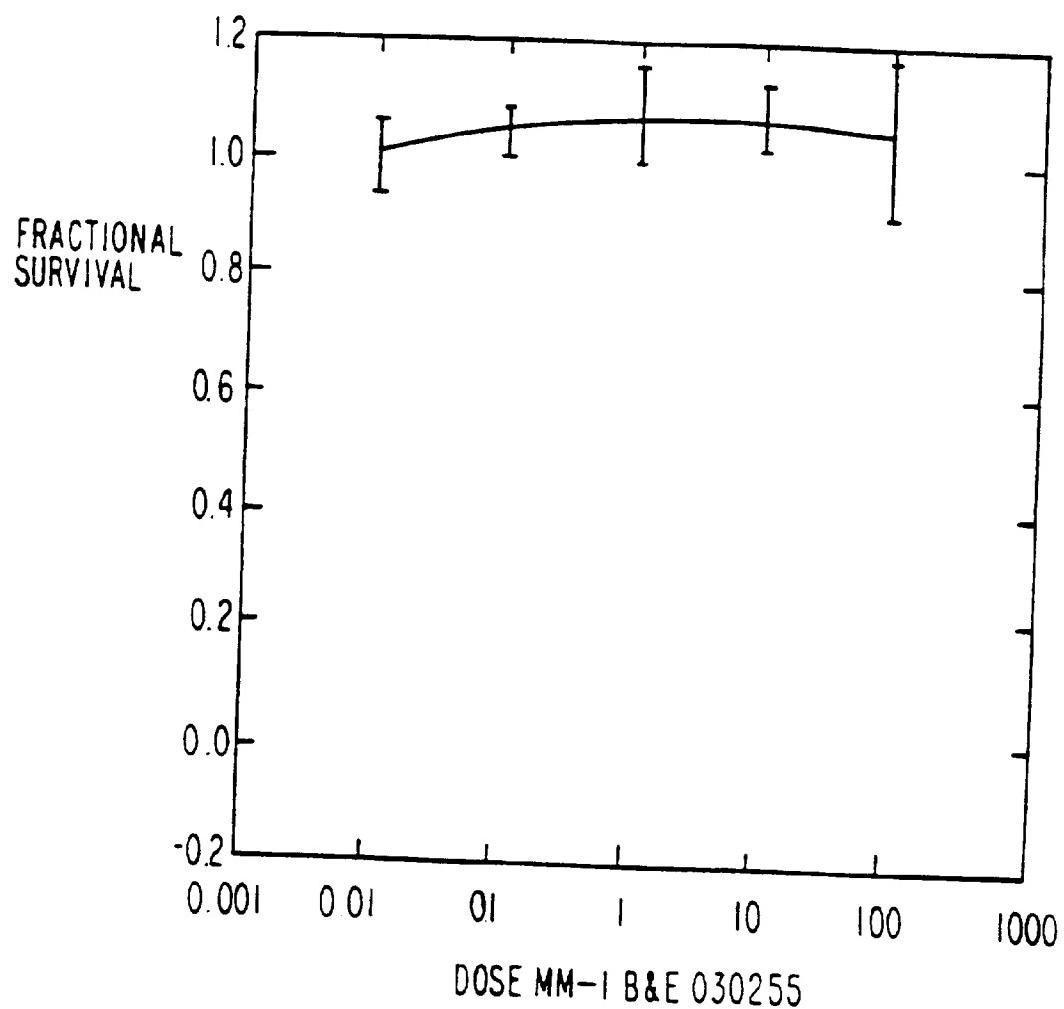


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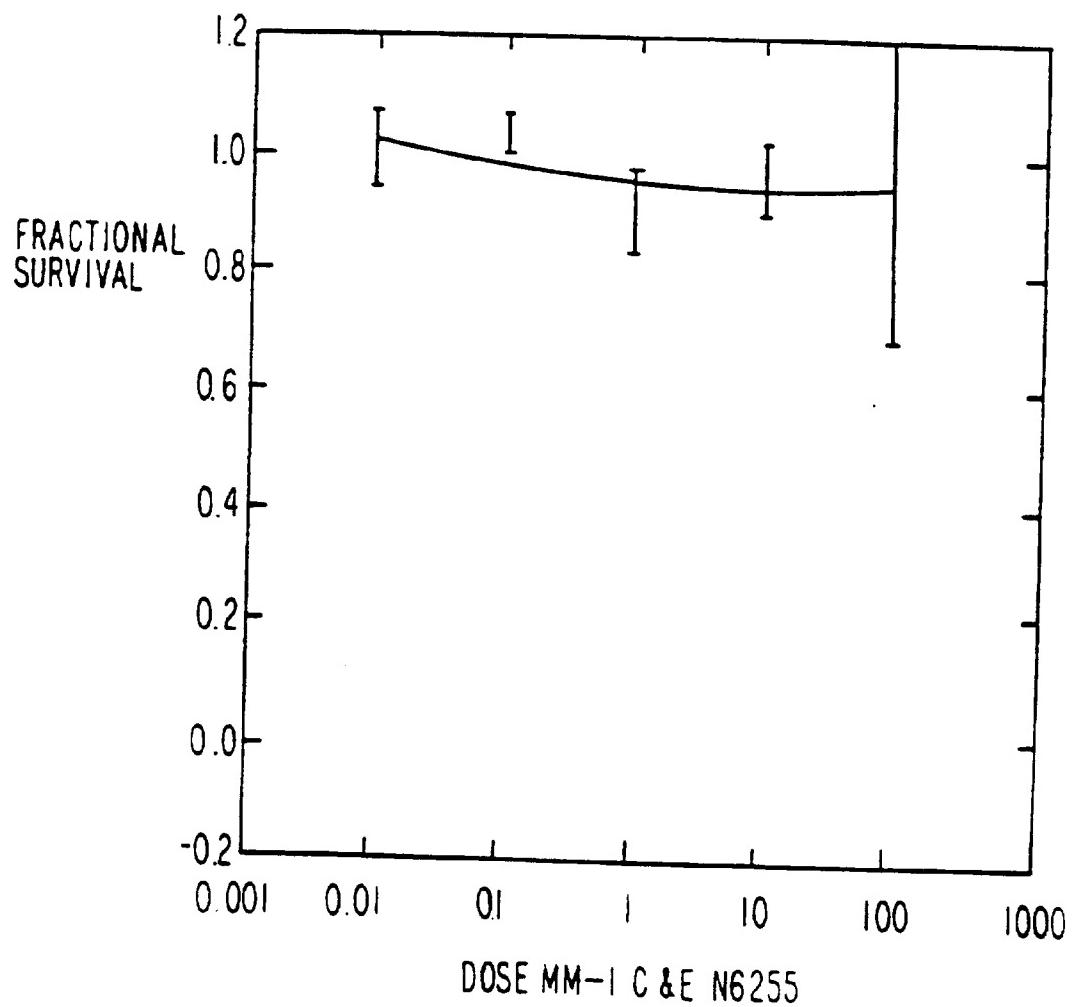
FIG. 11F



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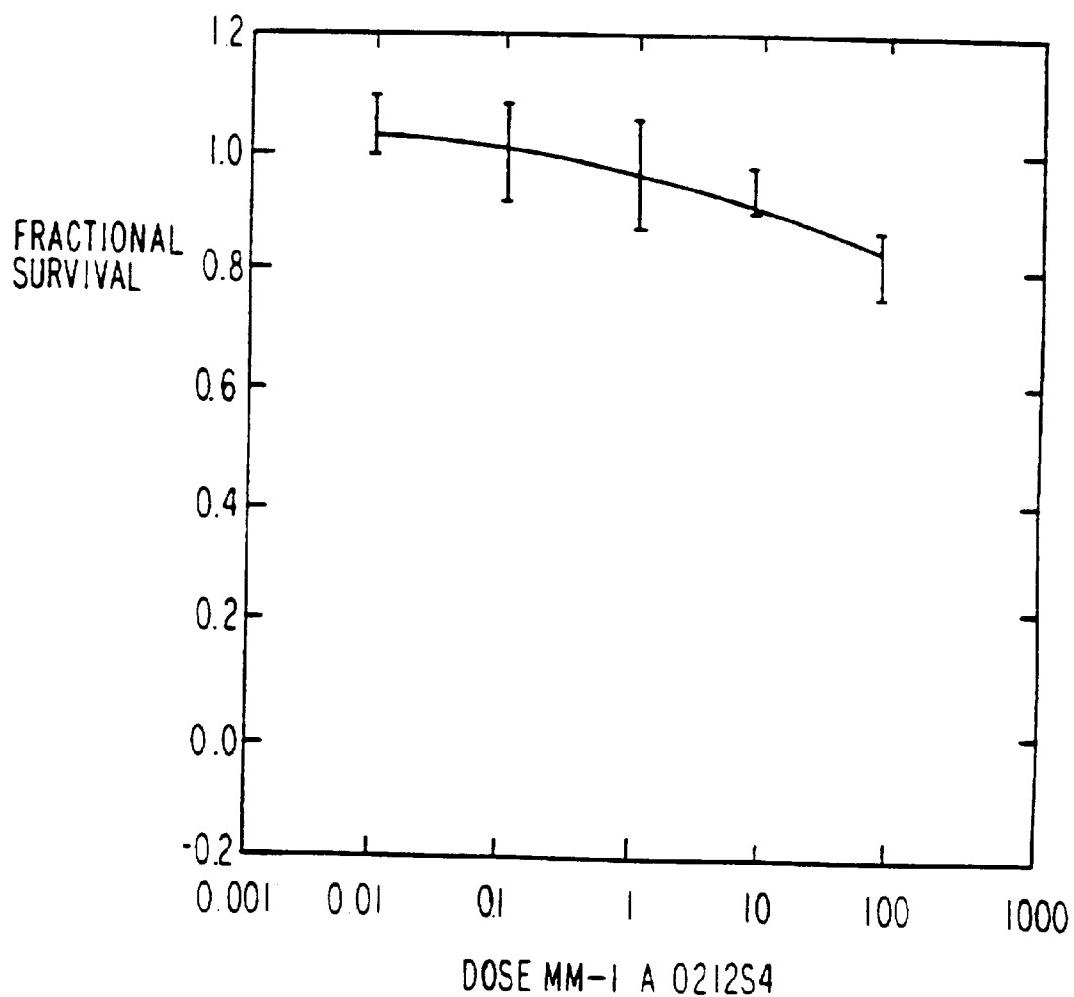
FIG. 11G

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FIG. IIIH

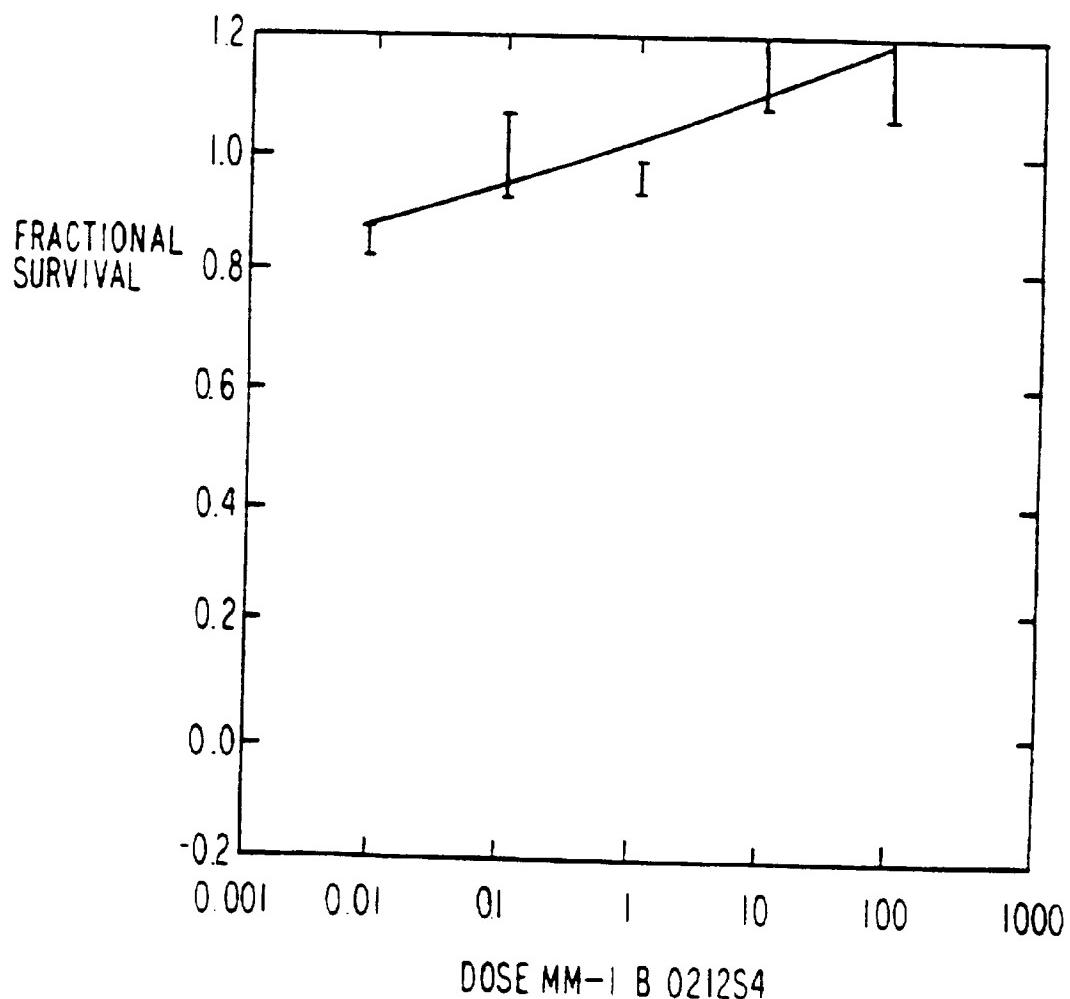
55/235

FIG. 12A



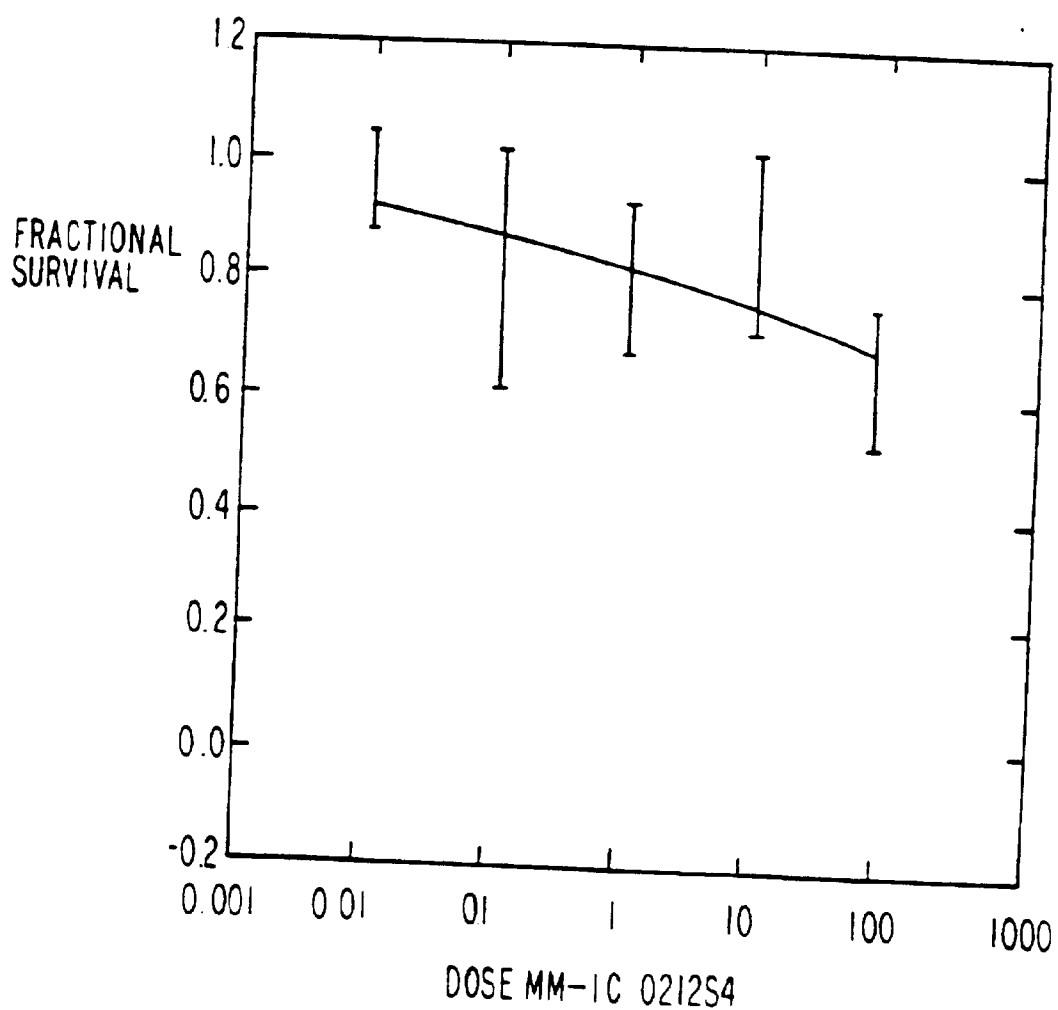
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FIG. 12B



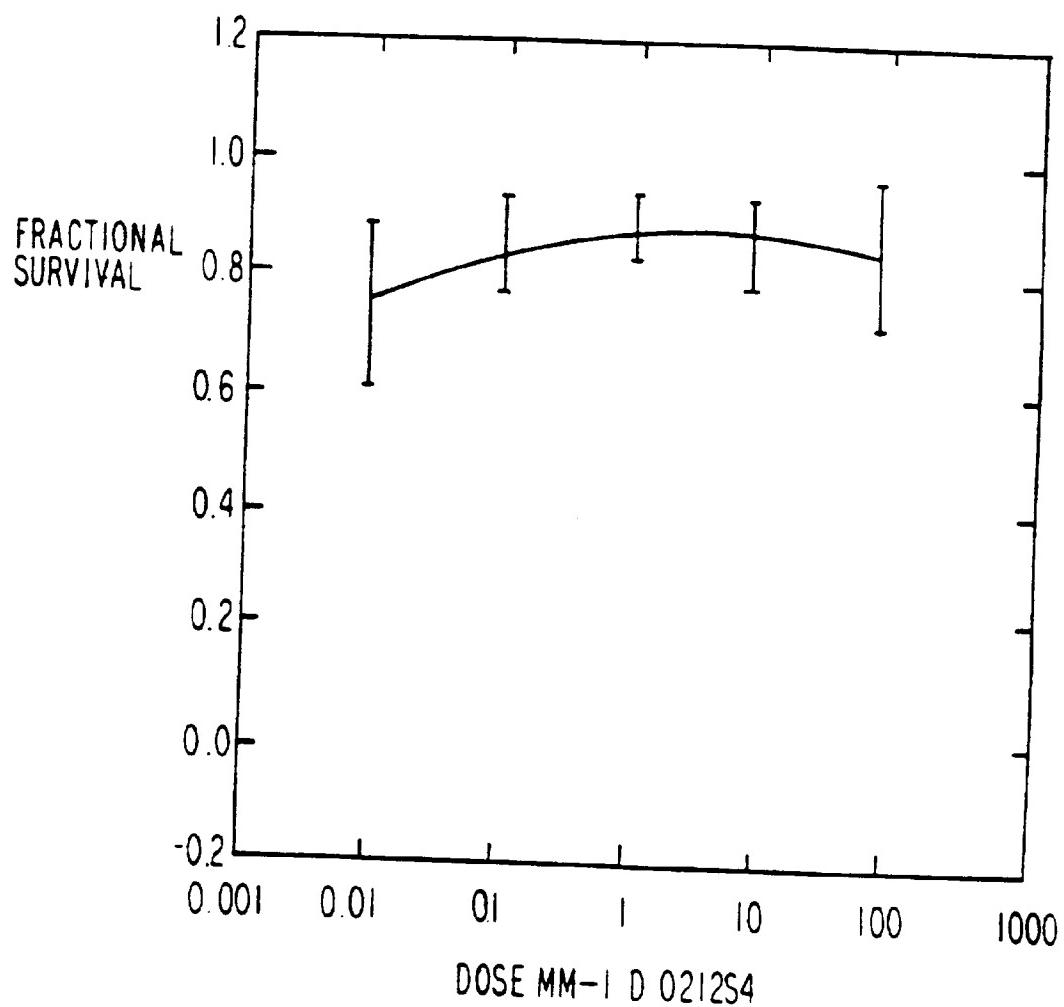
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FIG. 12C



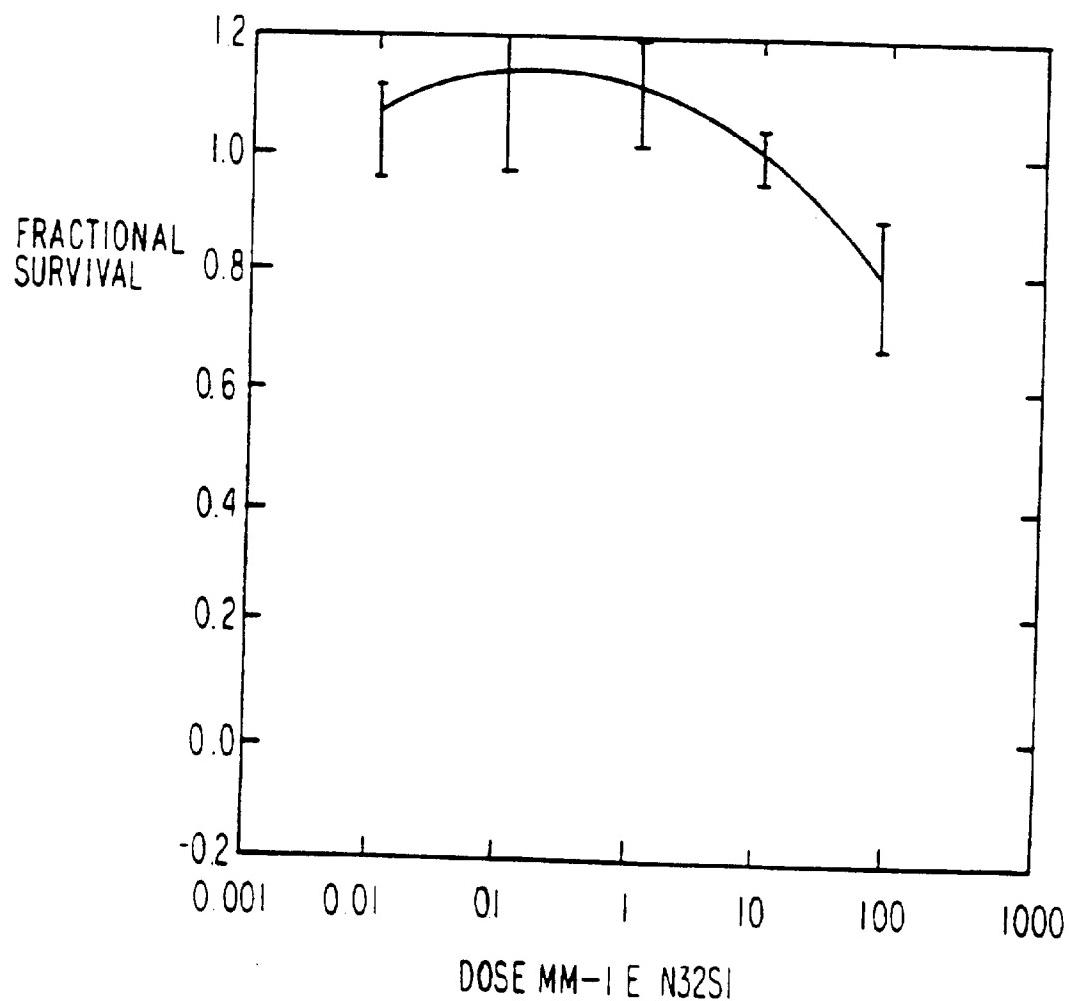
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FIG. 12D



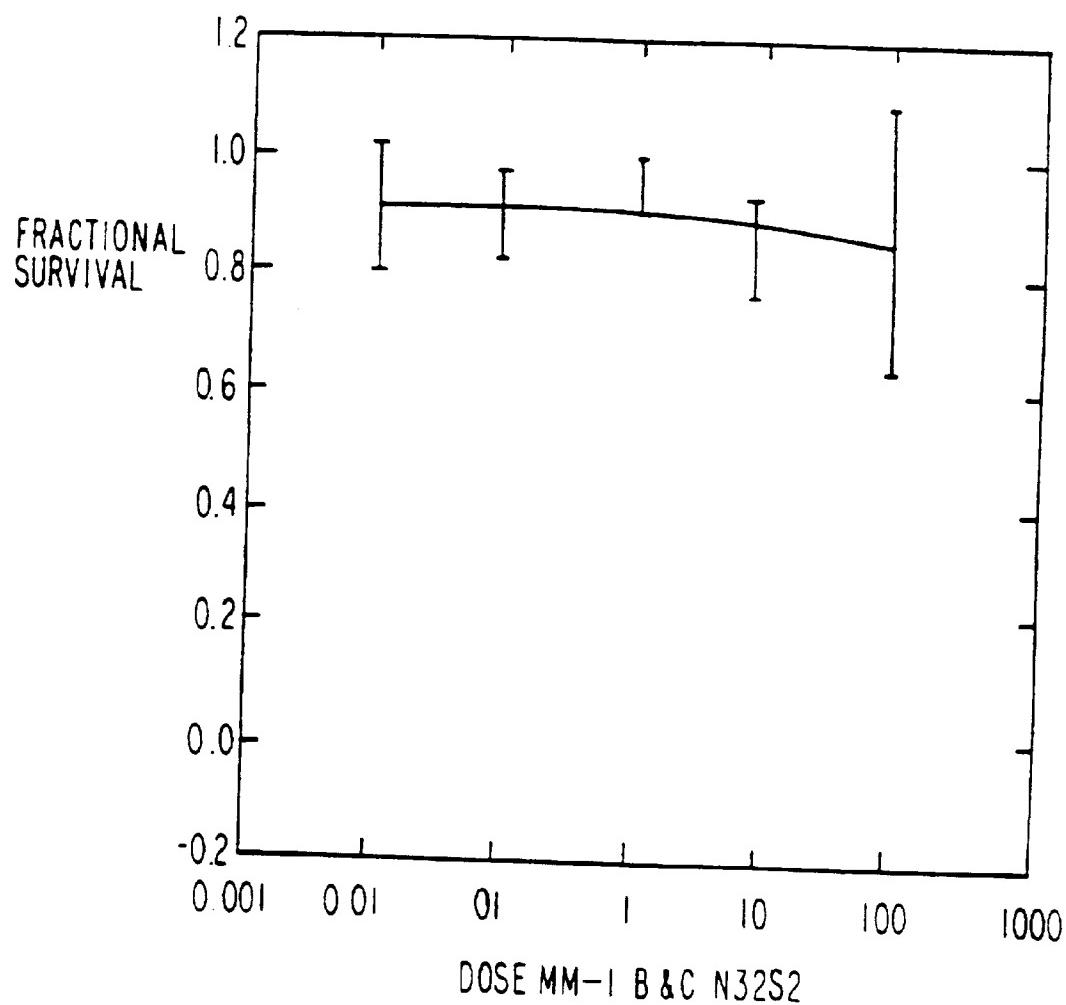
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FIG. 12E



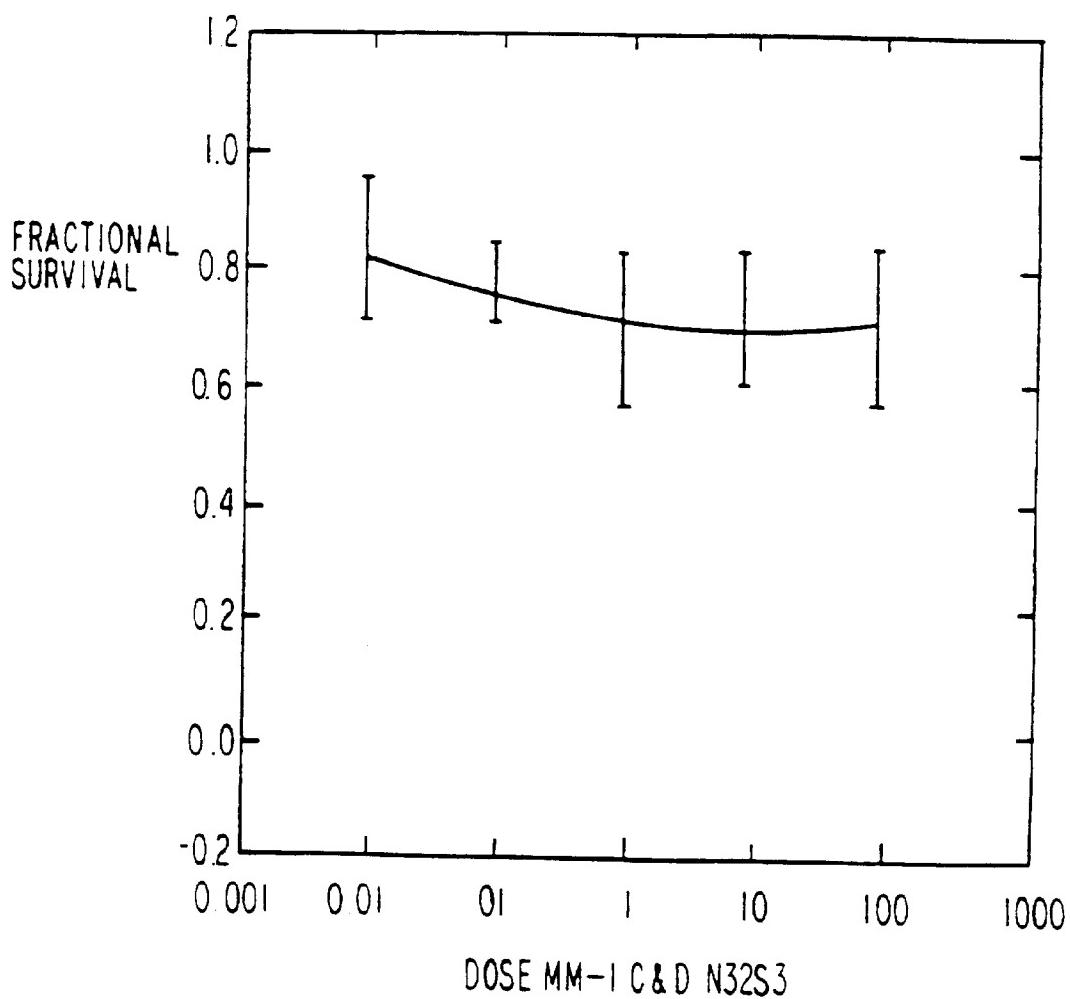
60/235

FIG. 12F

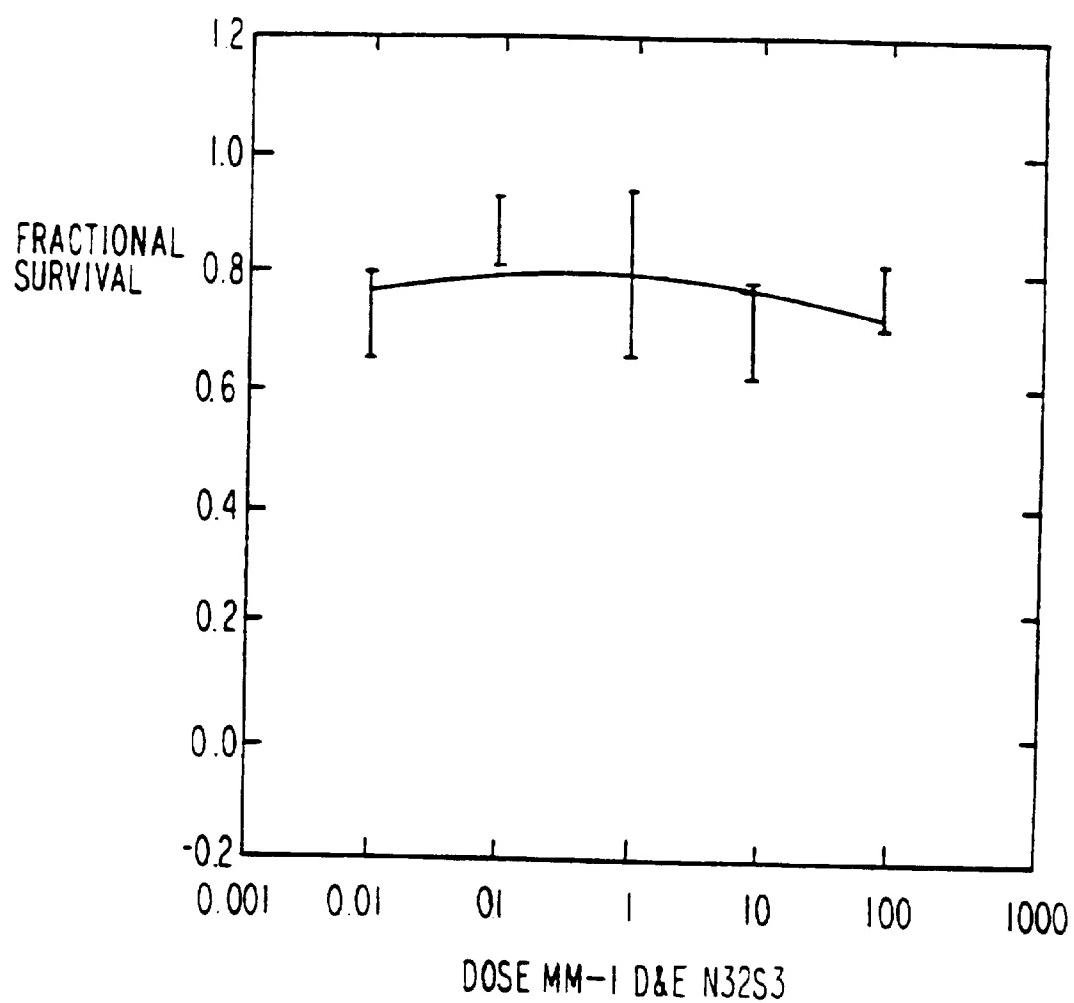


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FIG. 12G

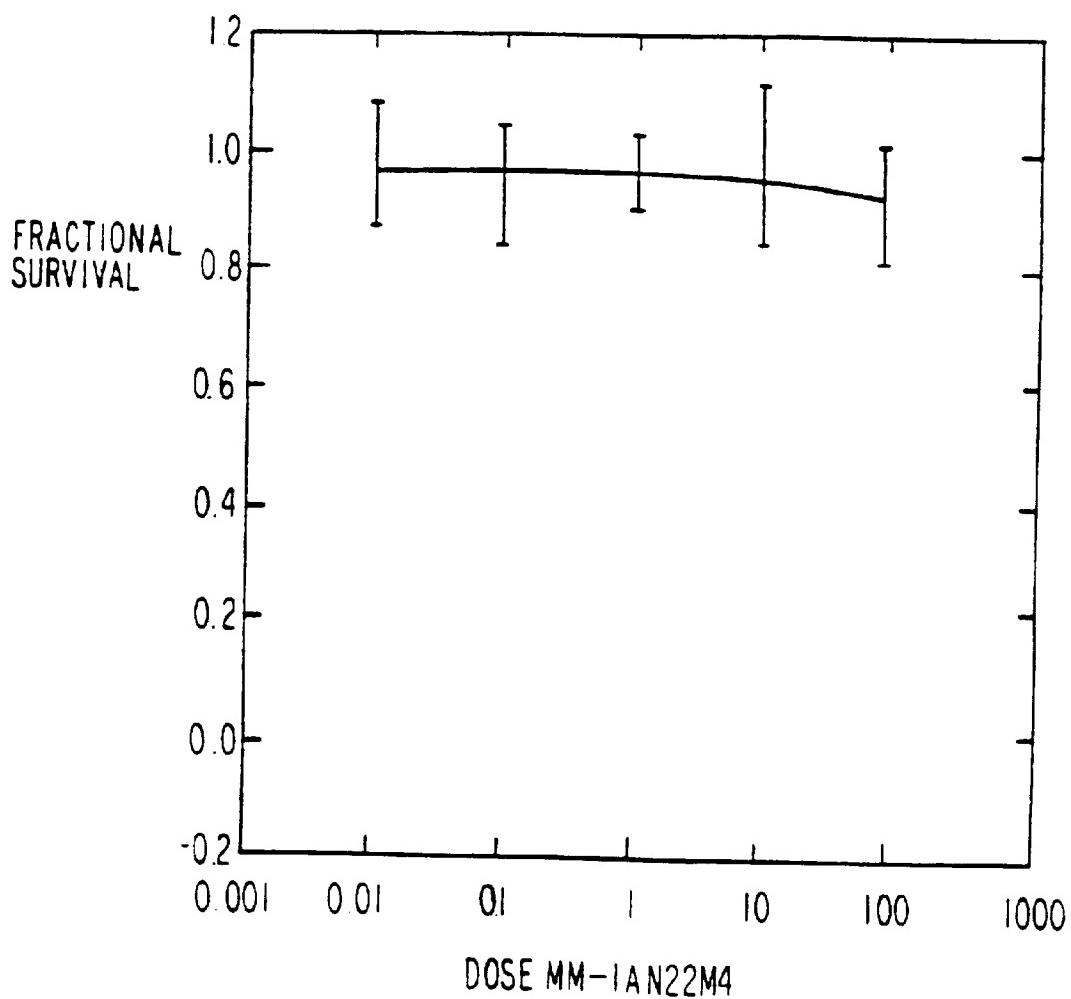


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FIG. 12H

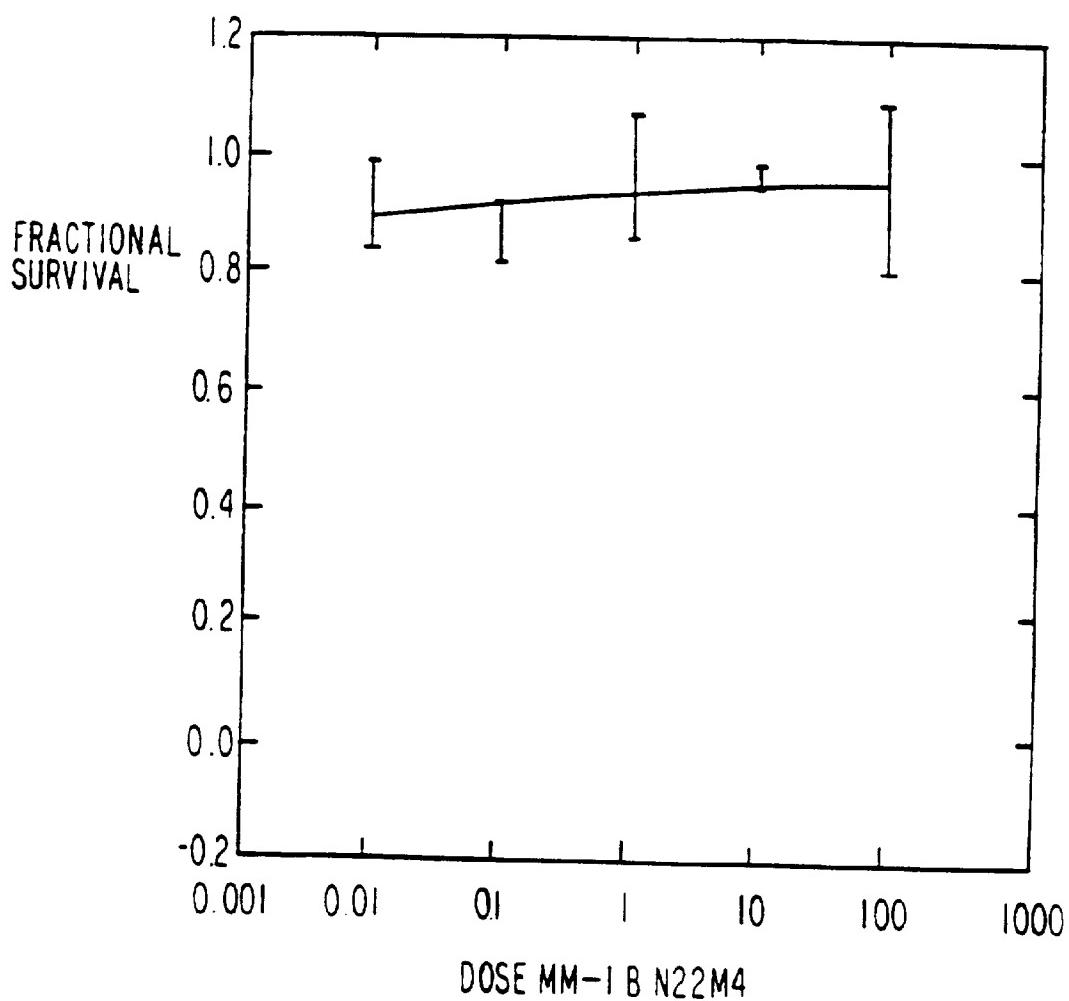
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FIG. 13A



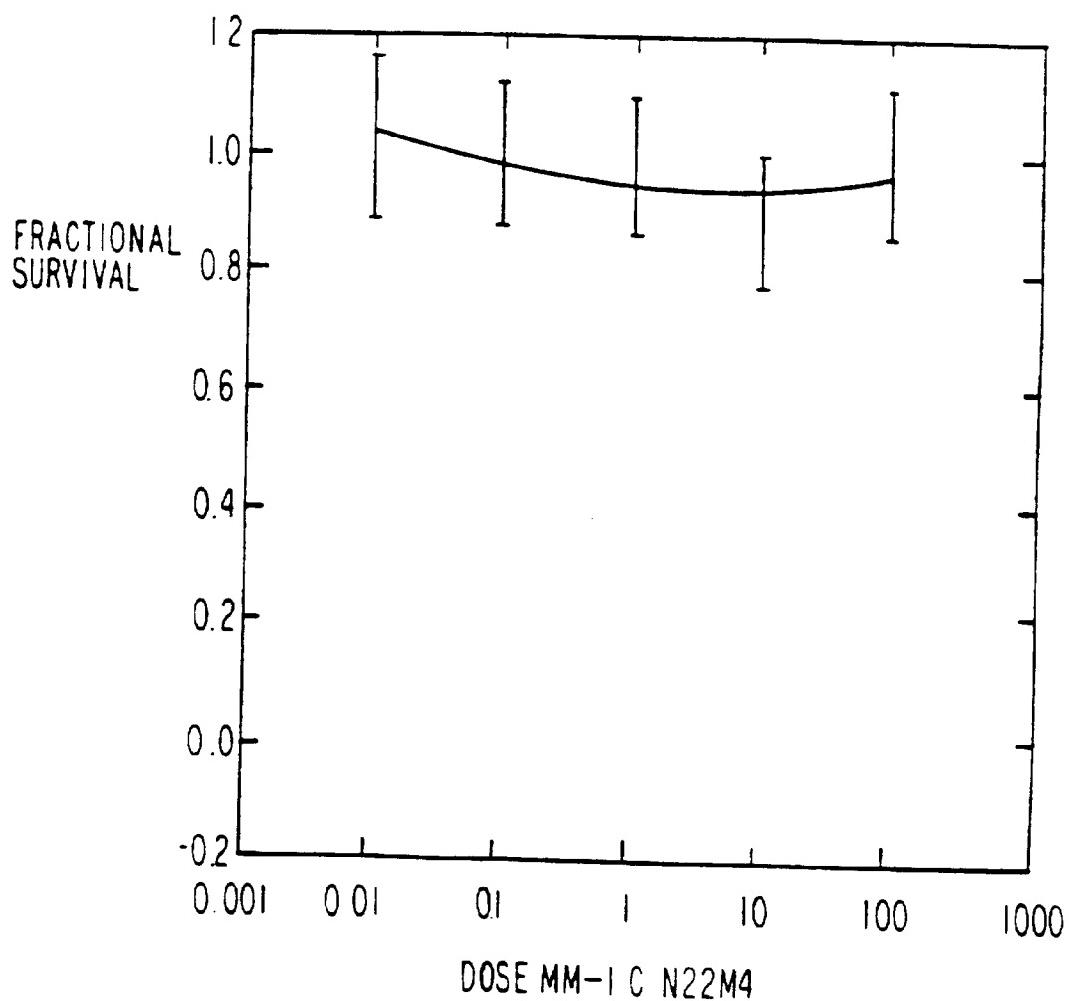
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FIG. 13B



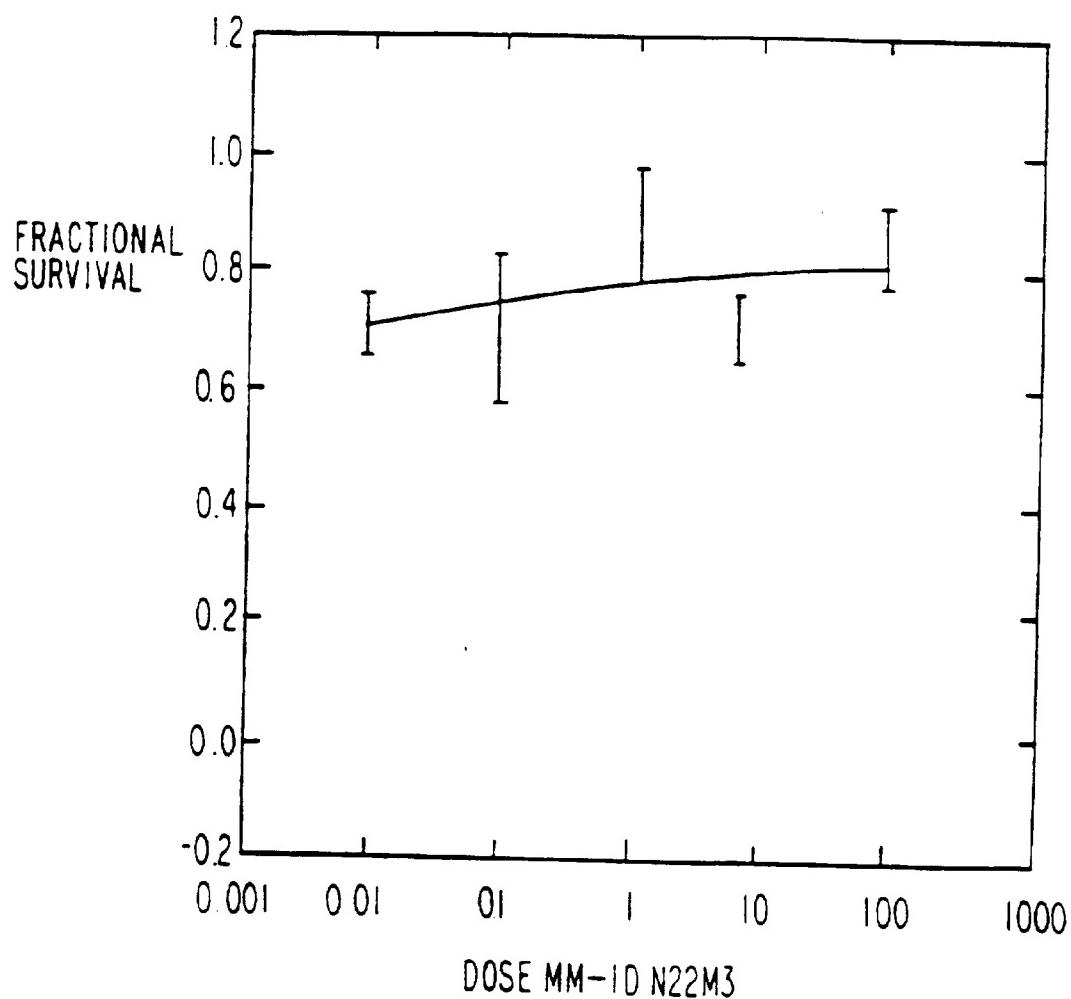
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FIG. 13C



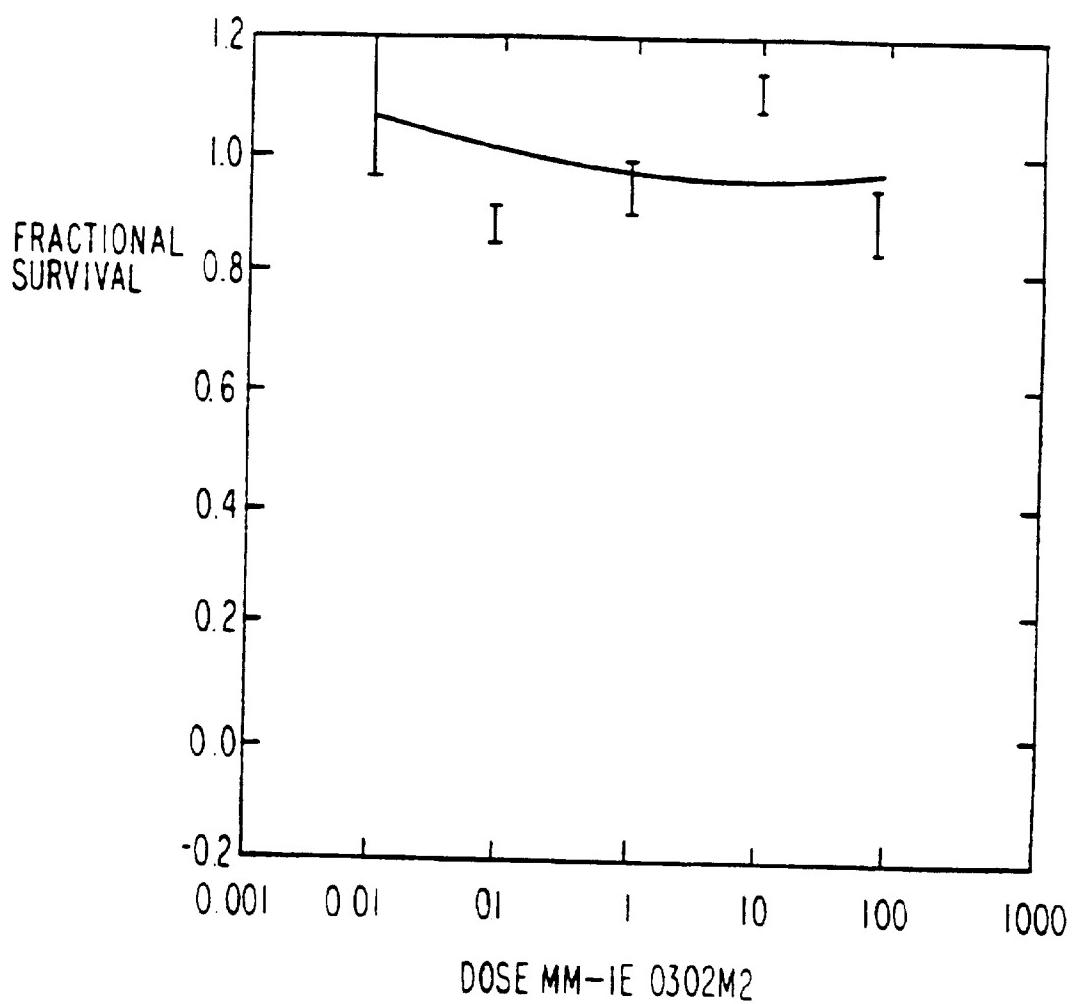
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FIG. 13D

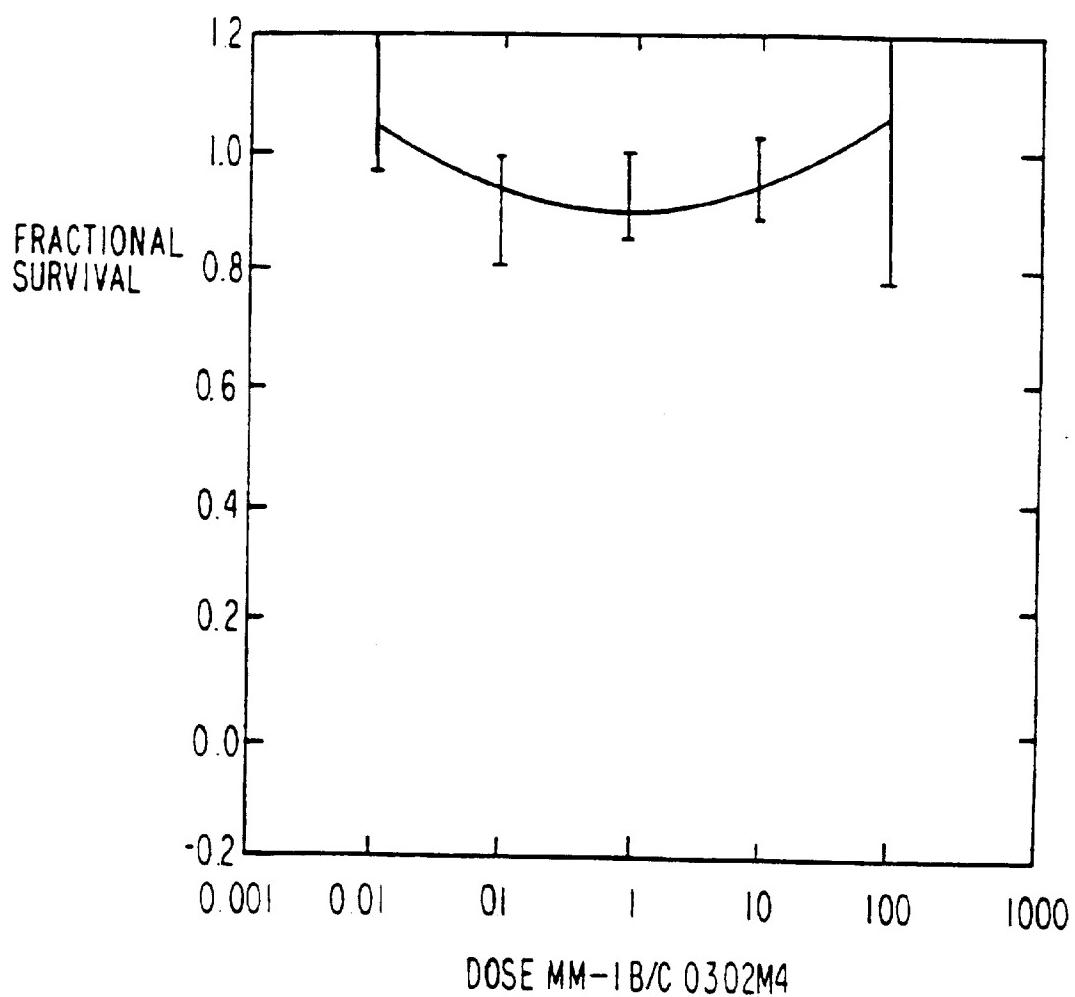


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FIG. 13E

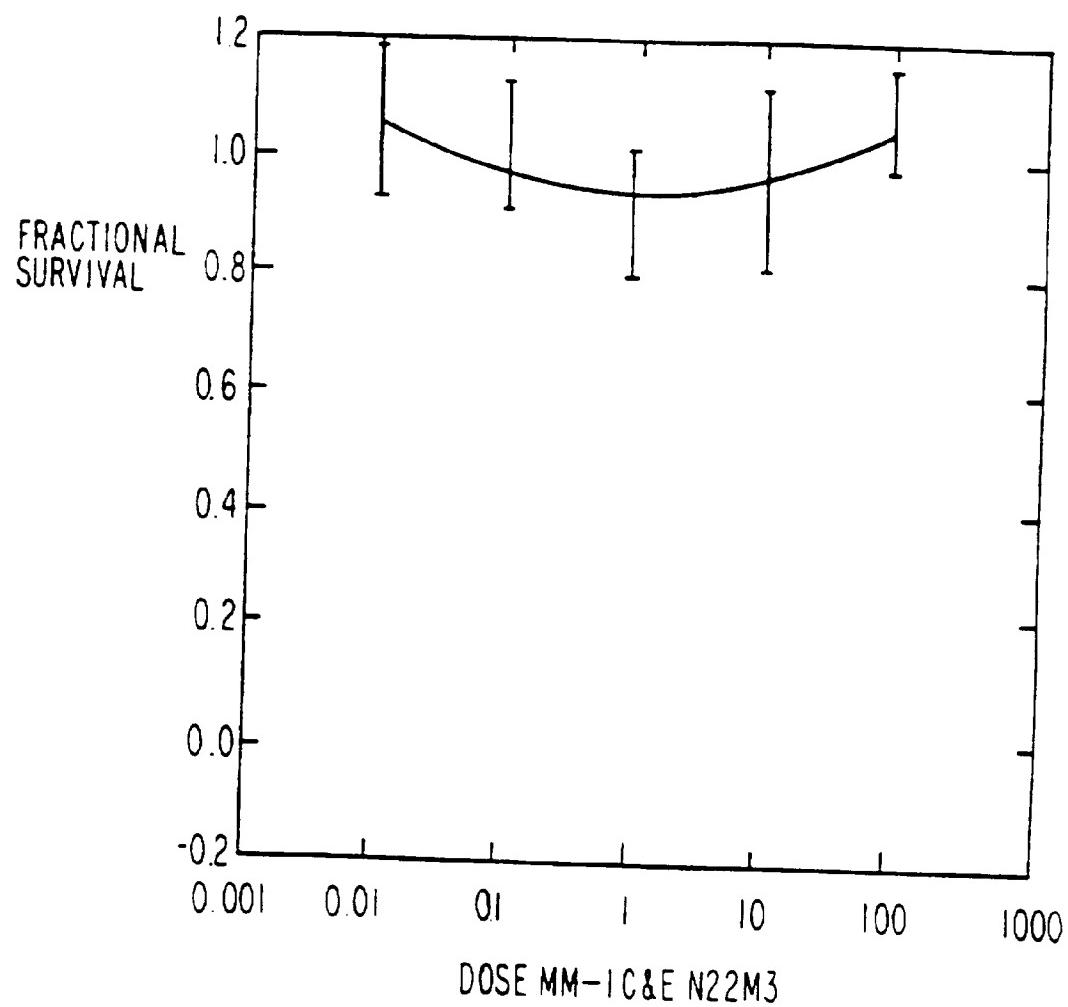


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FIG. 13F

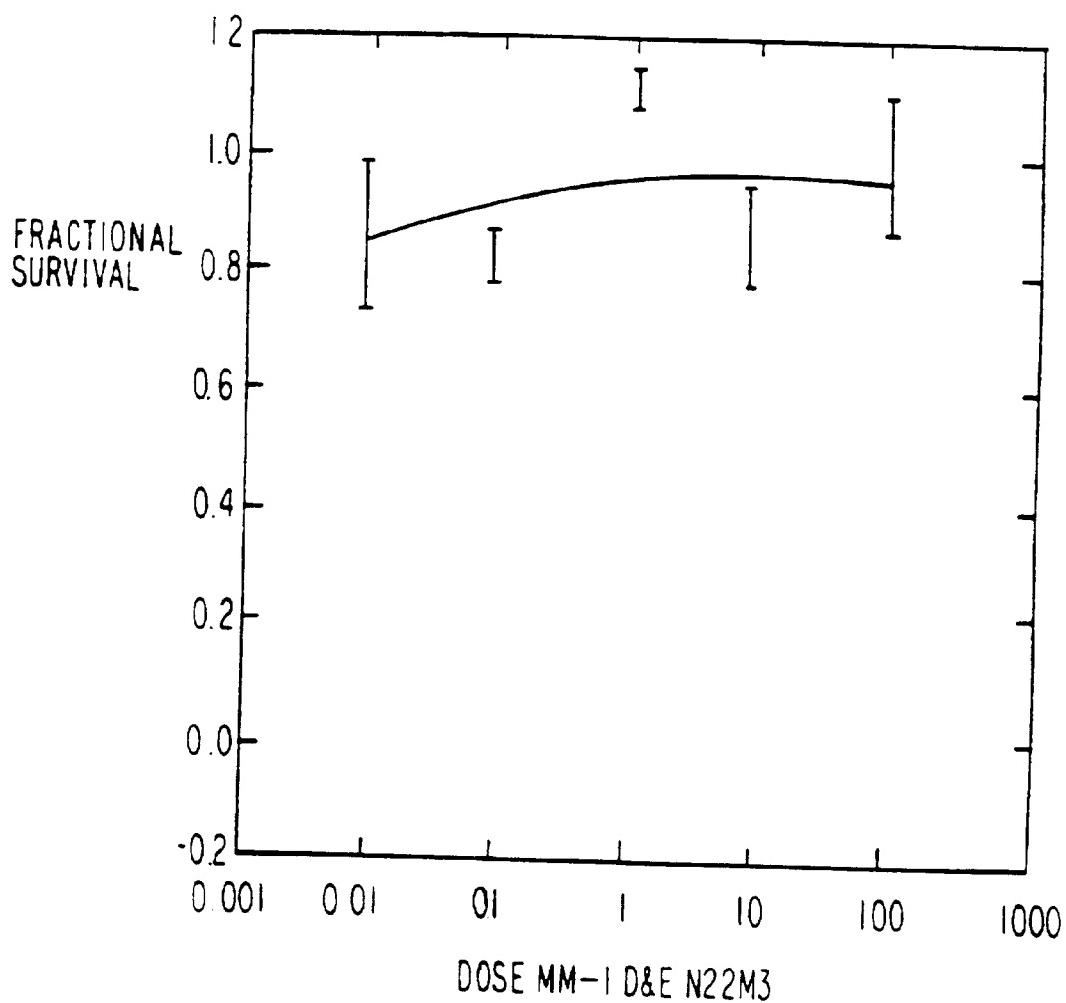
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FIG. 13G



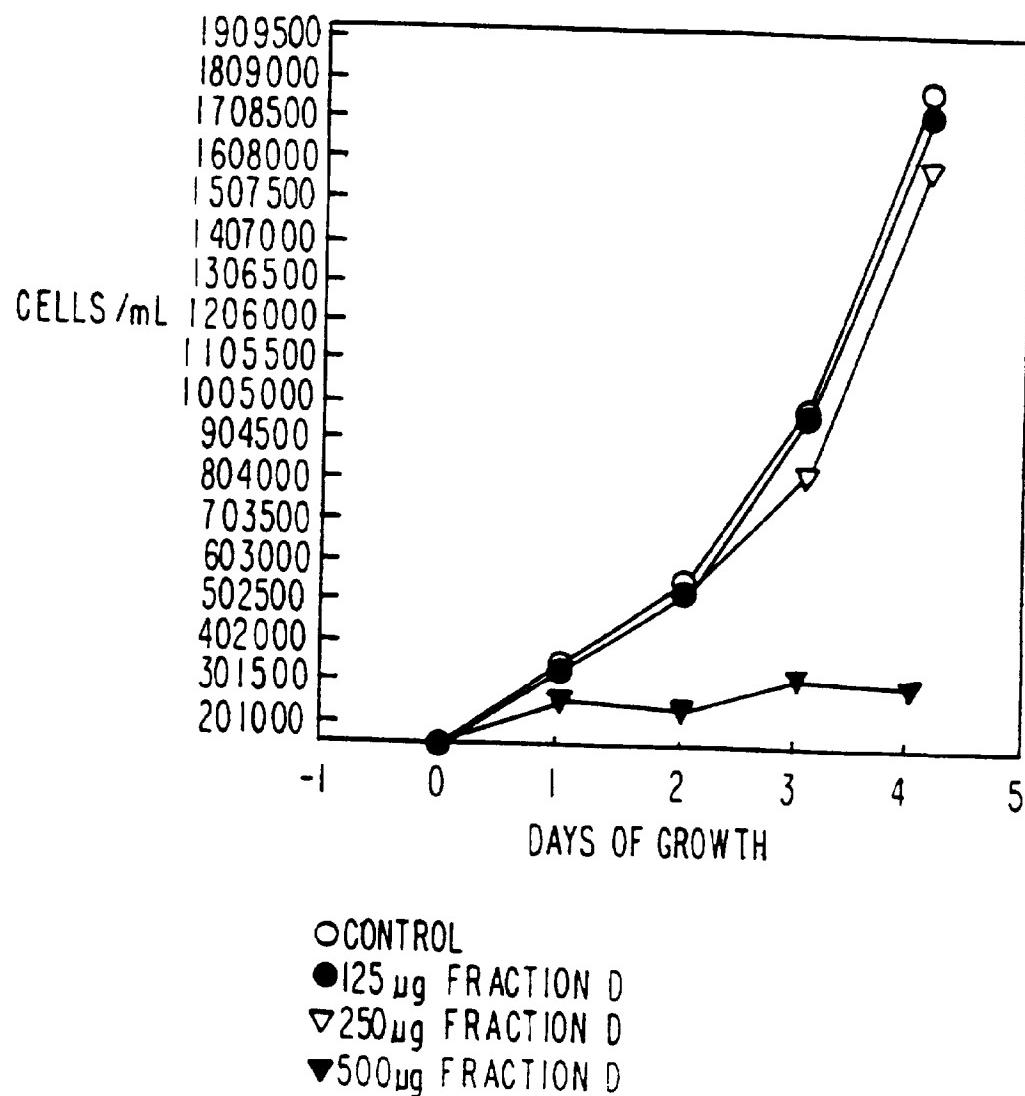
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FIG. 13H



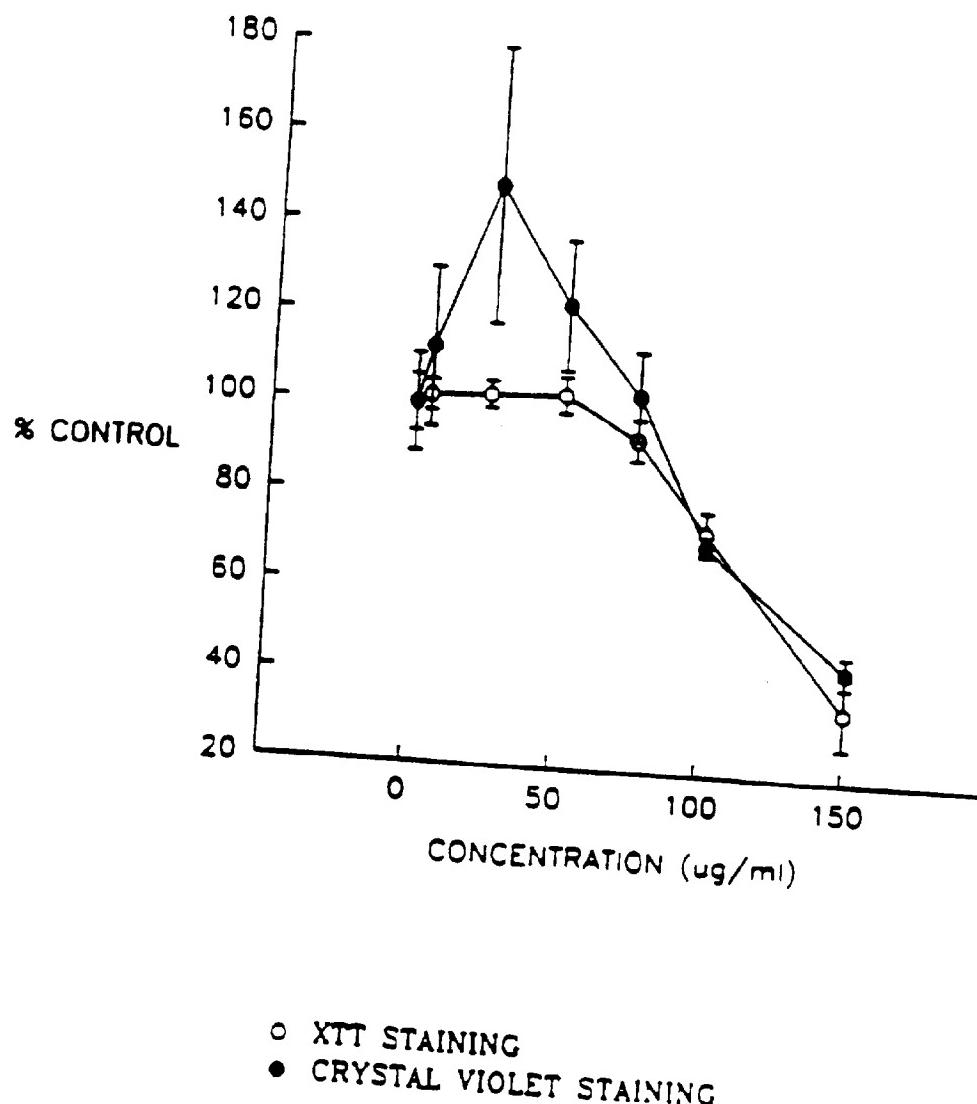
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FIG. 14



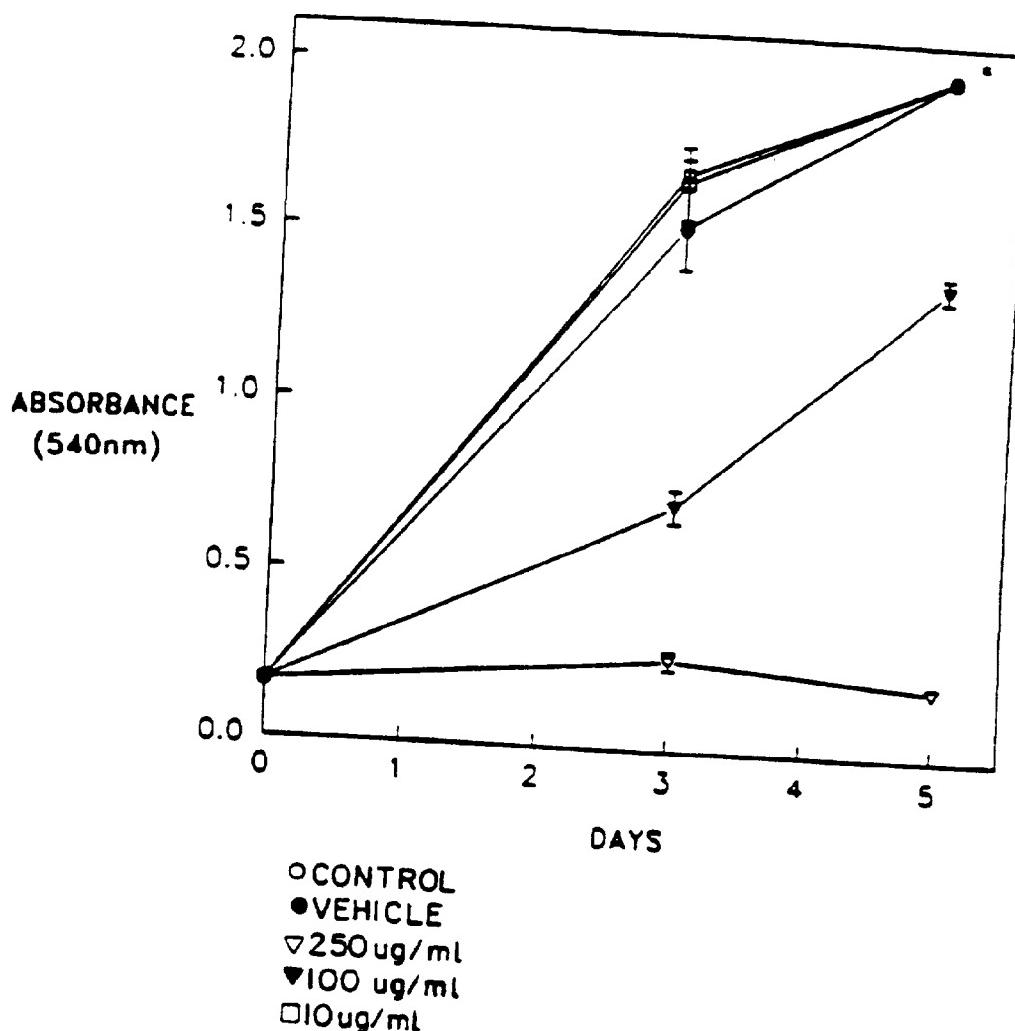
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FIG. 15A



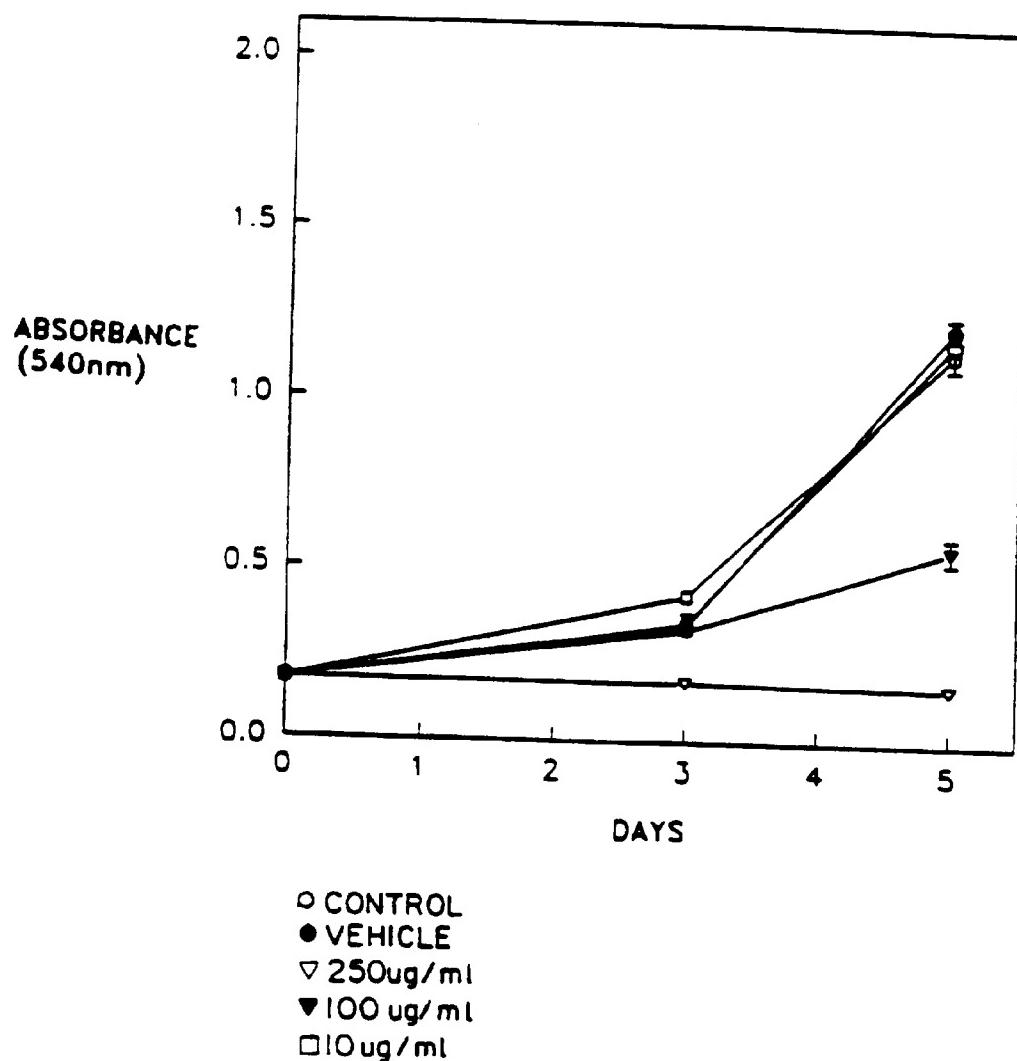
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FIG. 15B



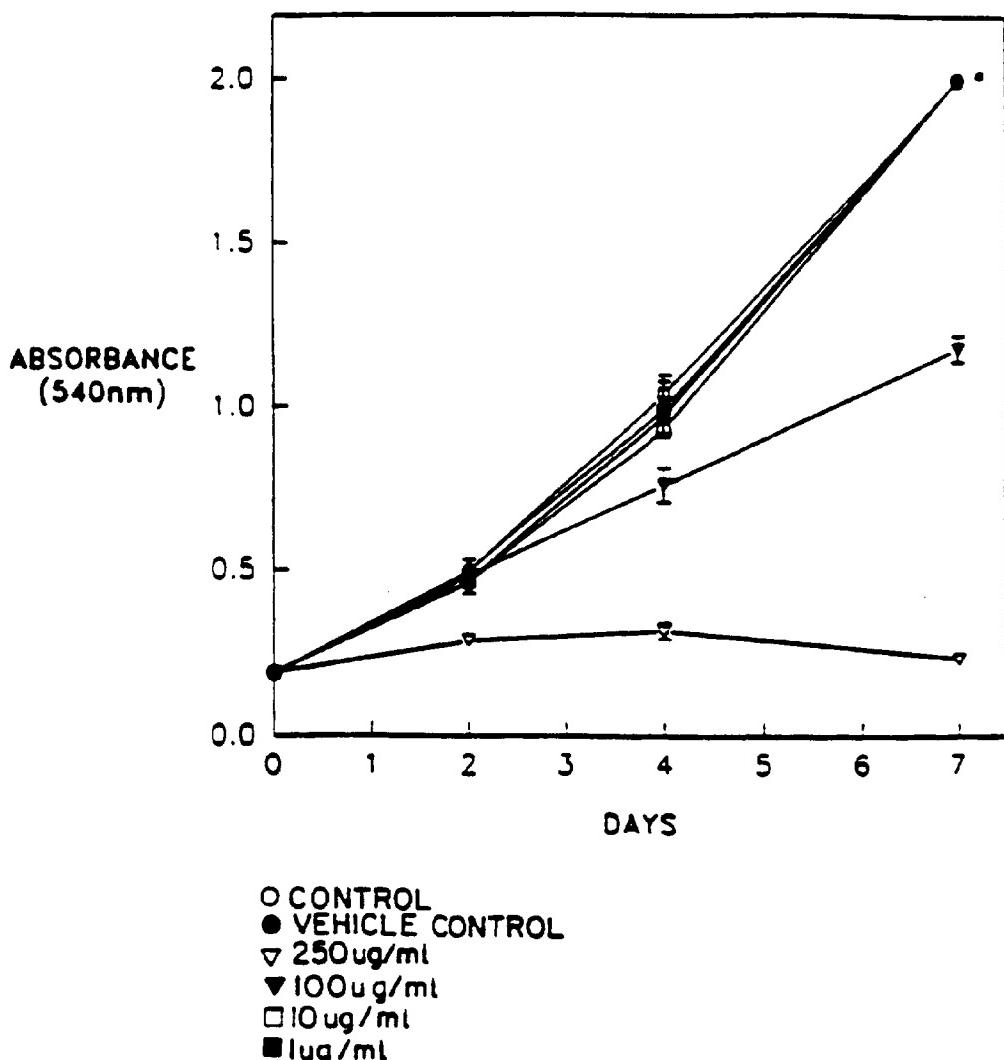
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FIG. 15C



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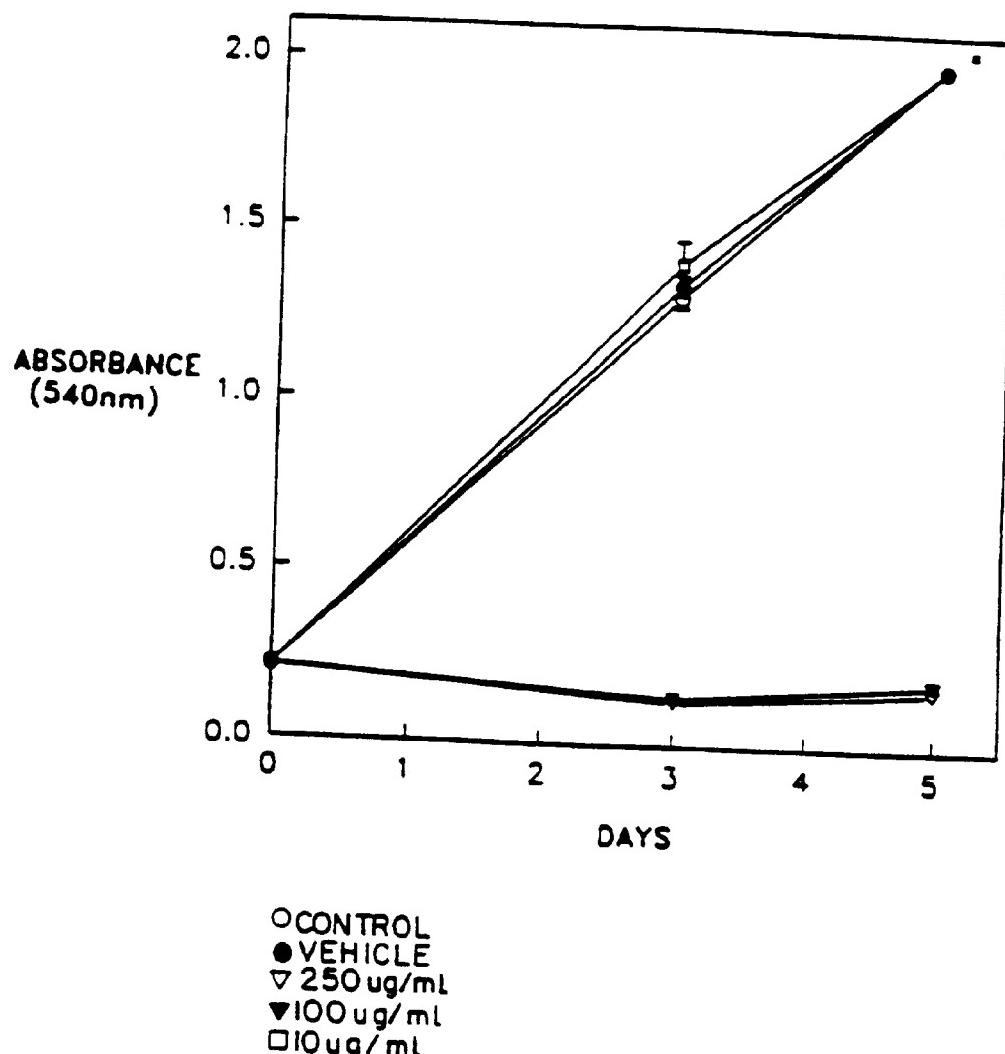
FIG. 15D



• NOTE: ABSORBANCE OF 2.0 INDICATES THE MAXIMUM ABSORBANCE OF THE PLATE READER. IT IS NOT REPRESENTATIVE OF CELL NUMBER.

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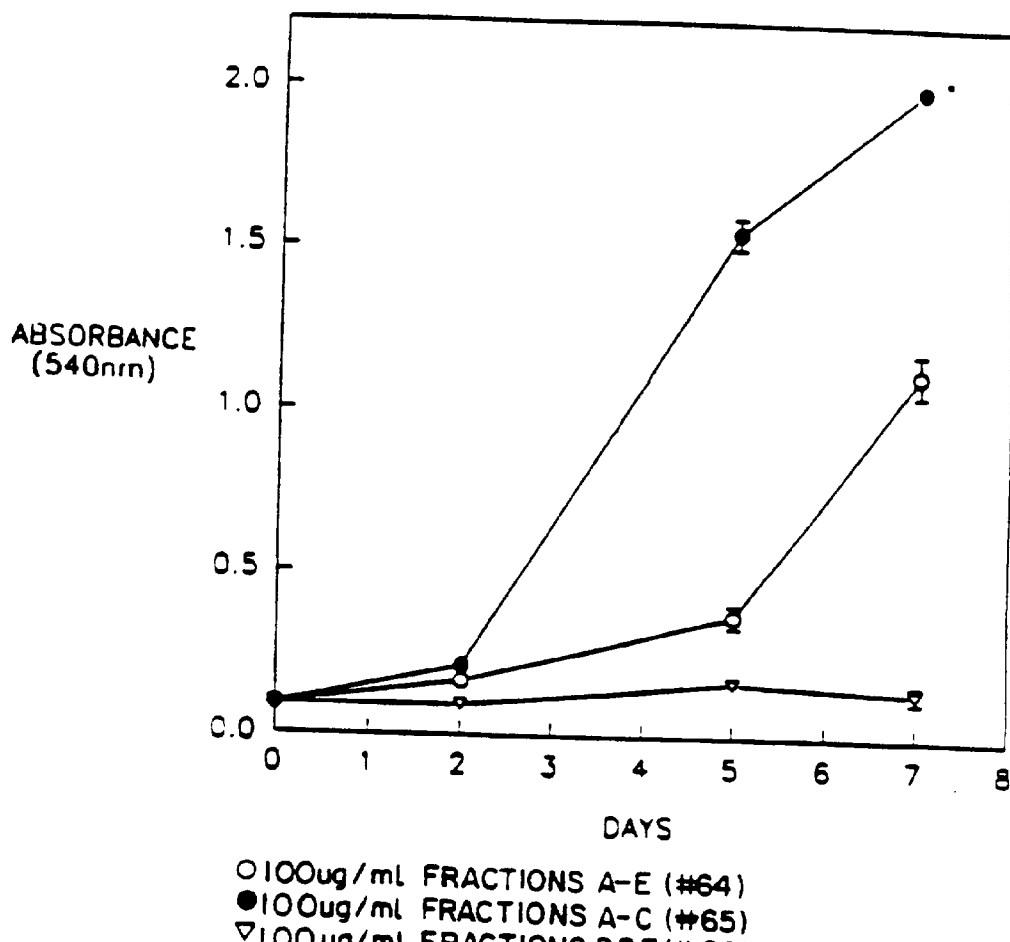
FIG. 15E



*NOTE: ABSORBANCE OF 2.0 INDICATES THE MAXIMUM ABSORBANCE OF THE PLATE READER. IT IS NOT REPRESENTATIVE OF CELL NUMBER.

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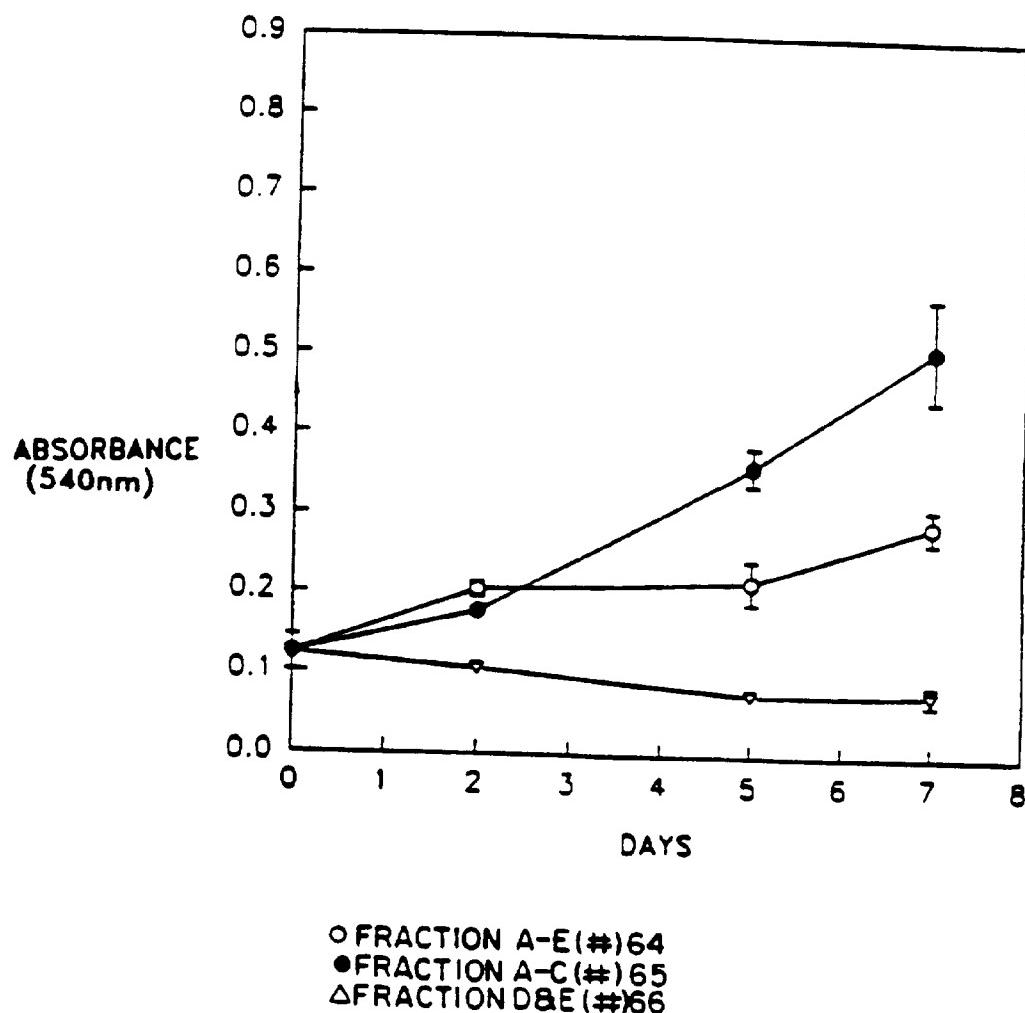
FIG. 15F



*NOTE: ABSORBANCE OF 2.0 INDICATES THE MAXIMUM ABSORBANCE OF THE PLATE READER. IT IS NOT REPRESENTATIVE OF CELL NUMBER.

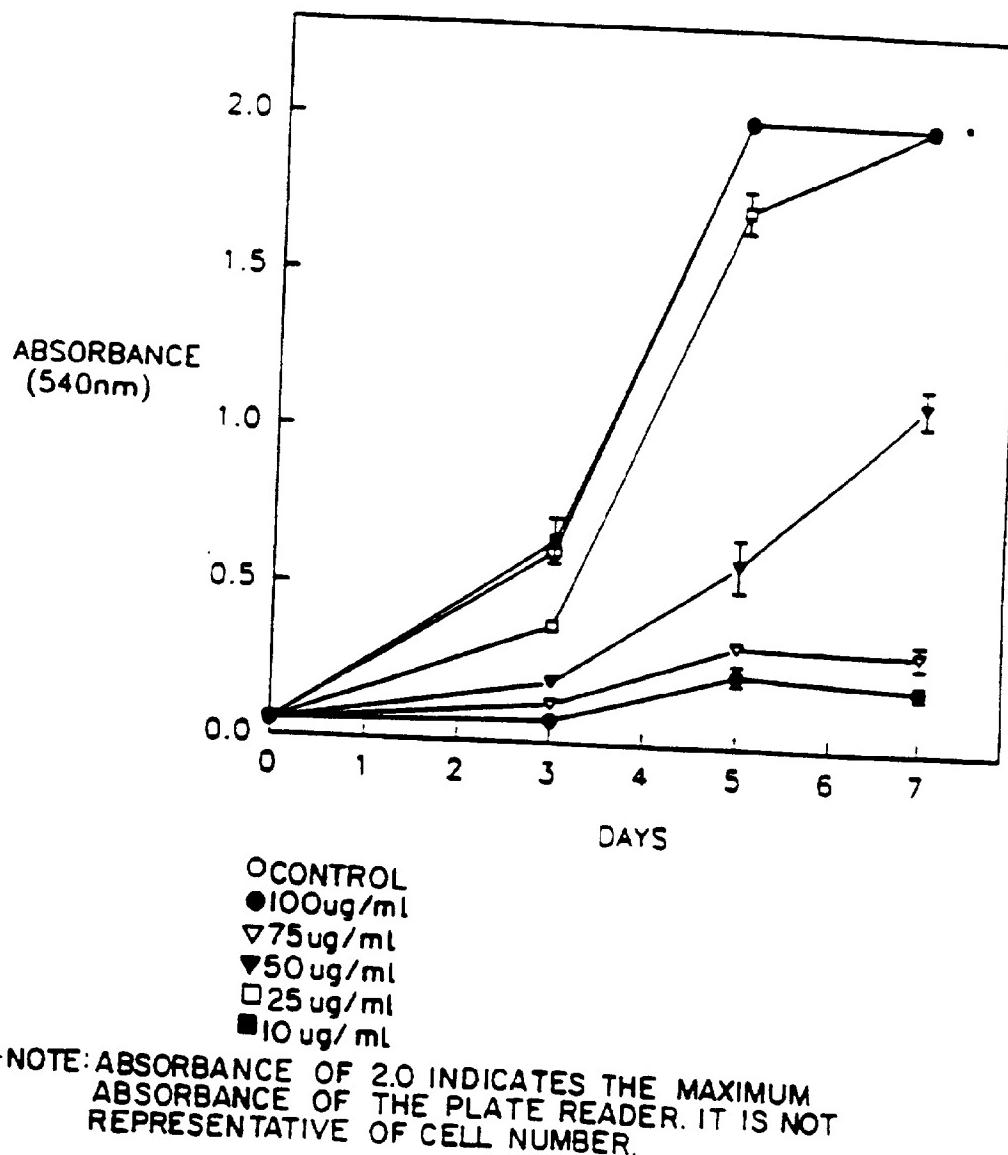
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FIG. 15G



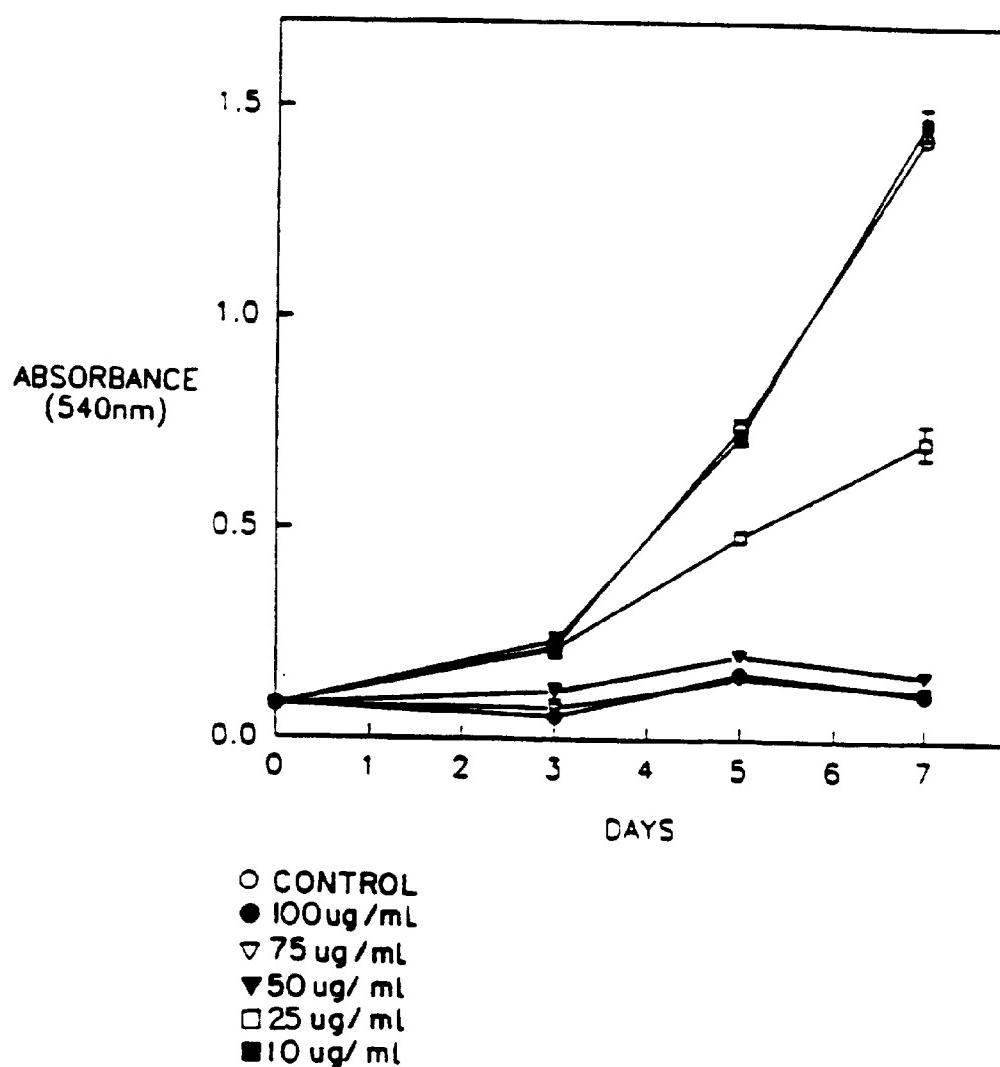
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FIG. 15H



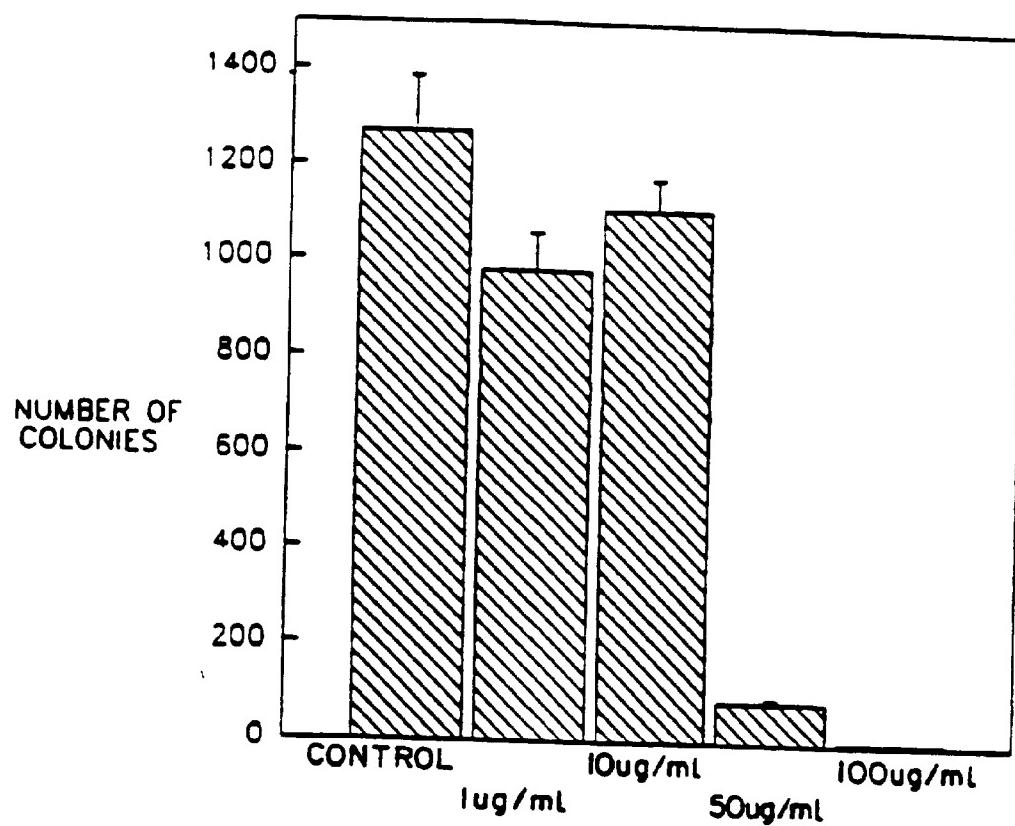
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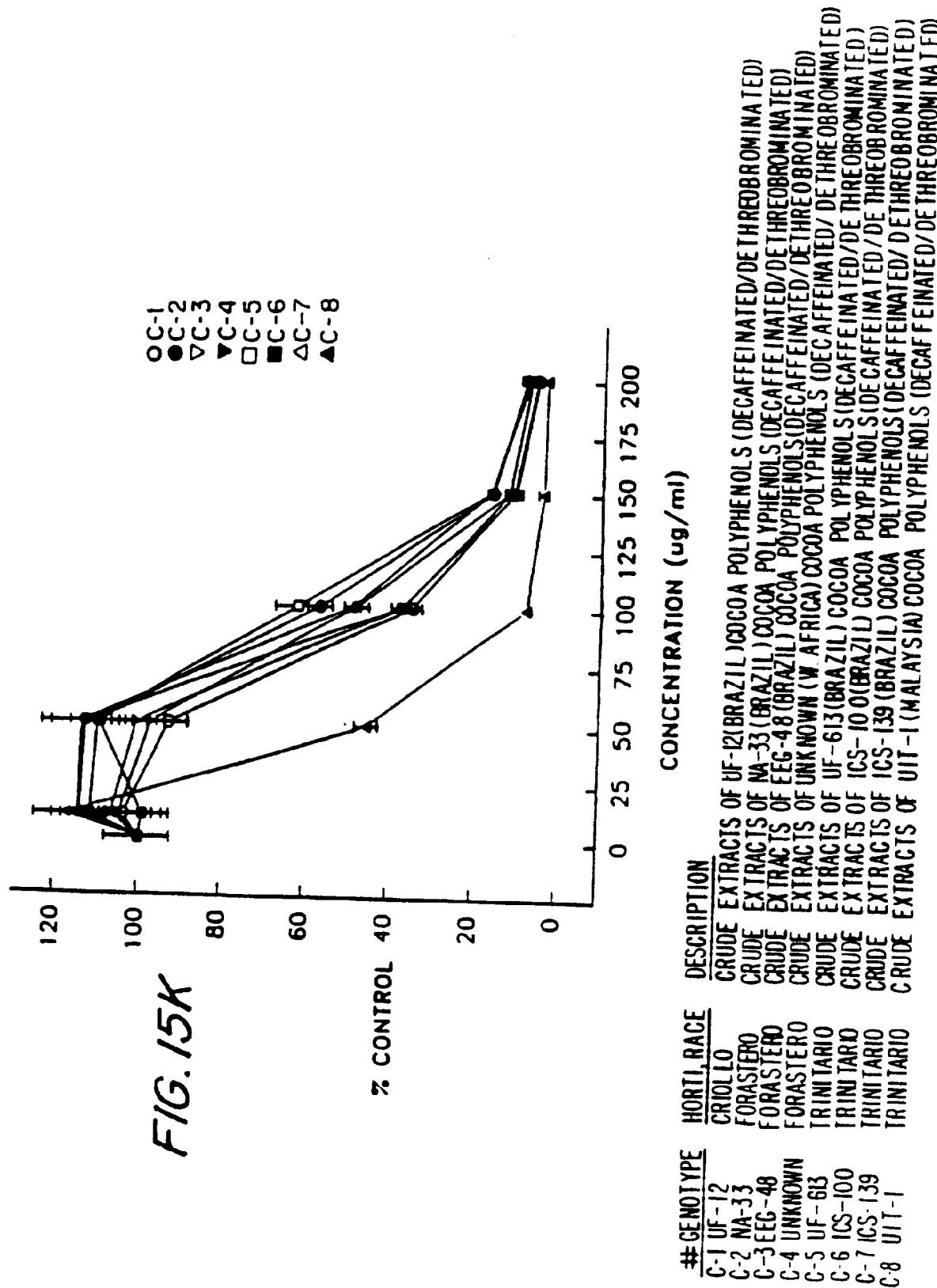
FIG. 15I



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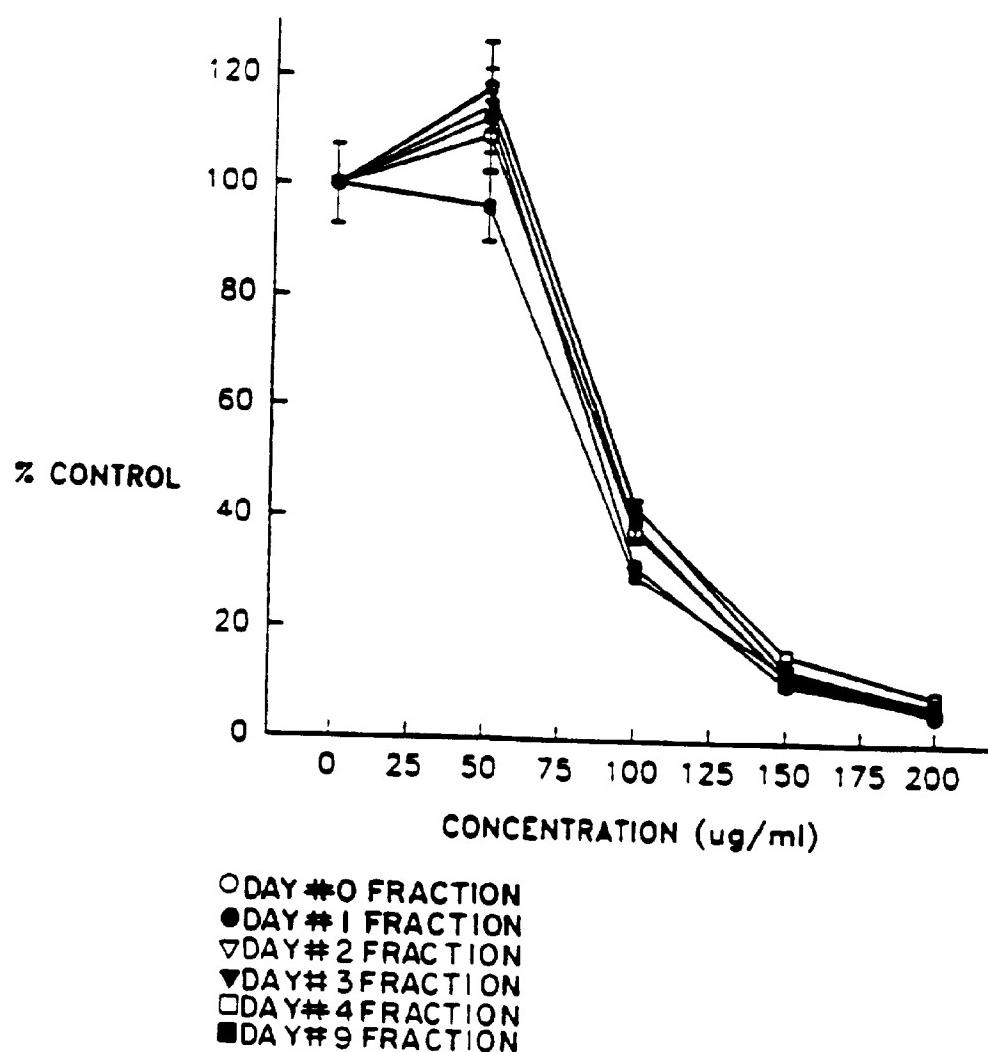
FIG. 15J





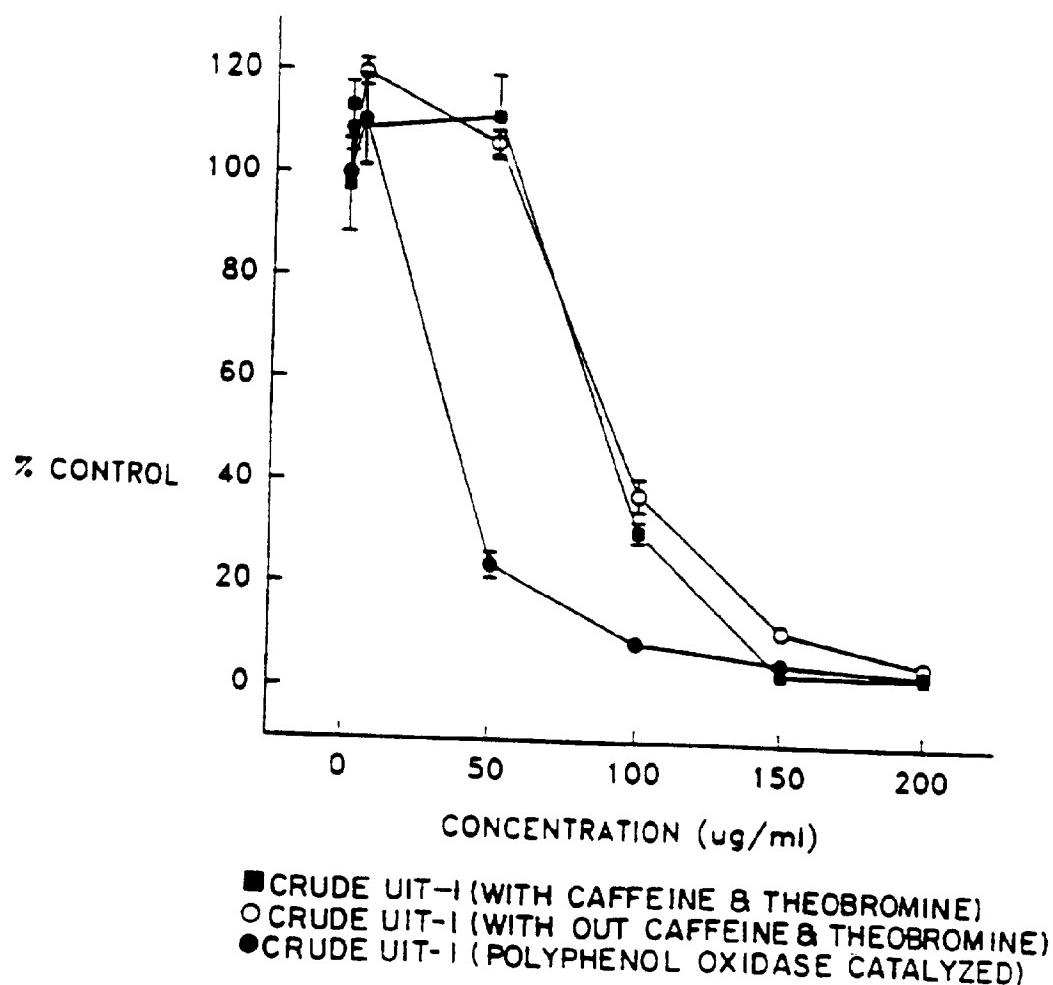
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FIG. 15L



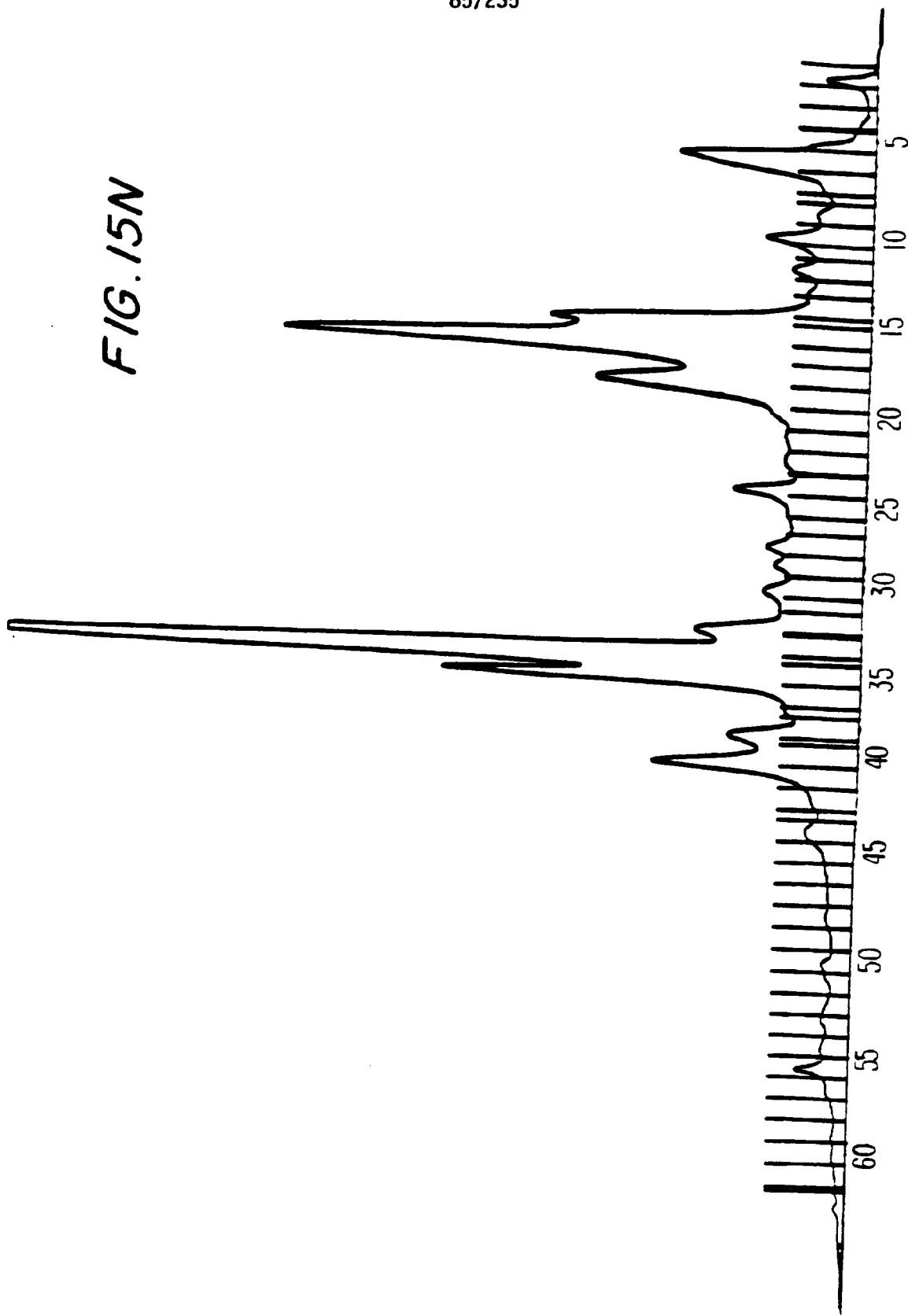
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FIG. 15M



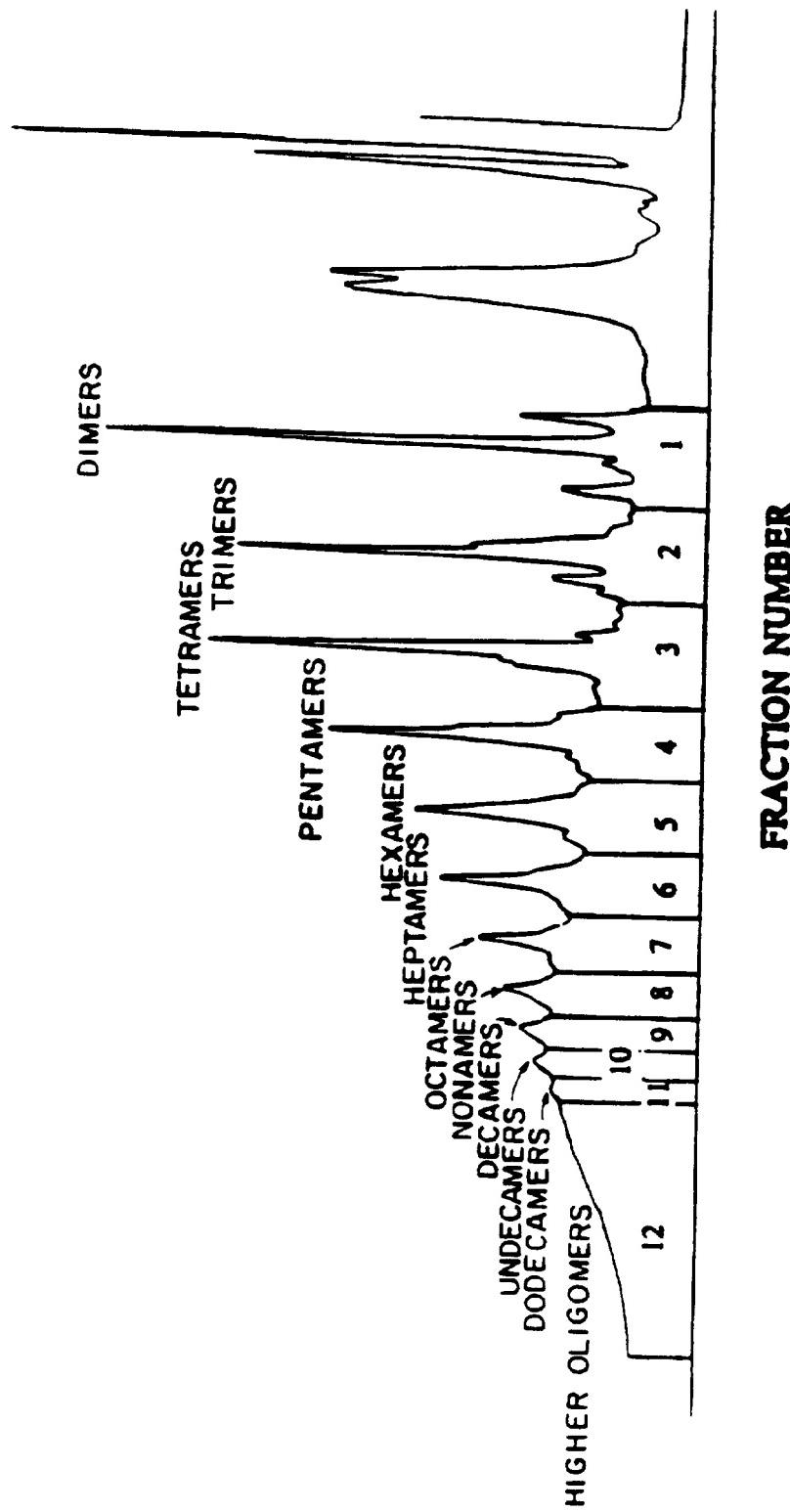
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FIG. 15N



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FIG. 150



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FIG. 16

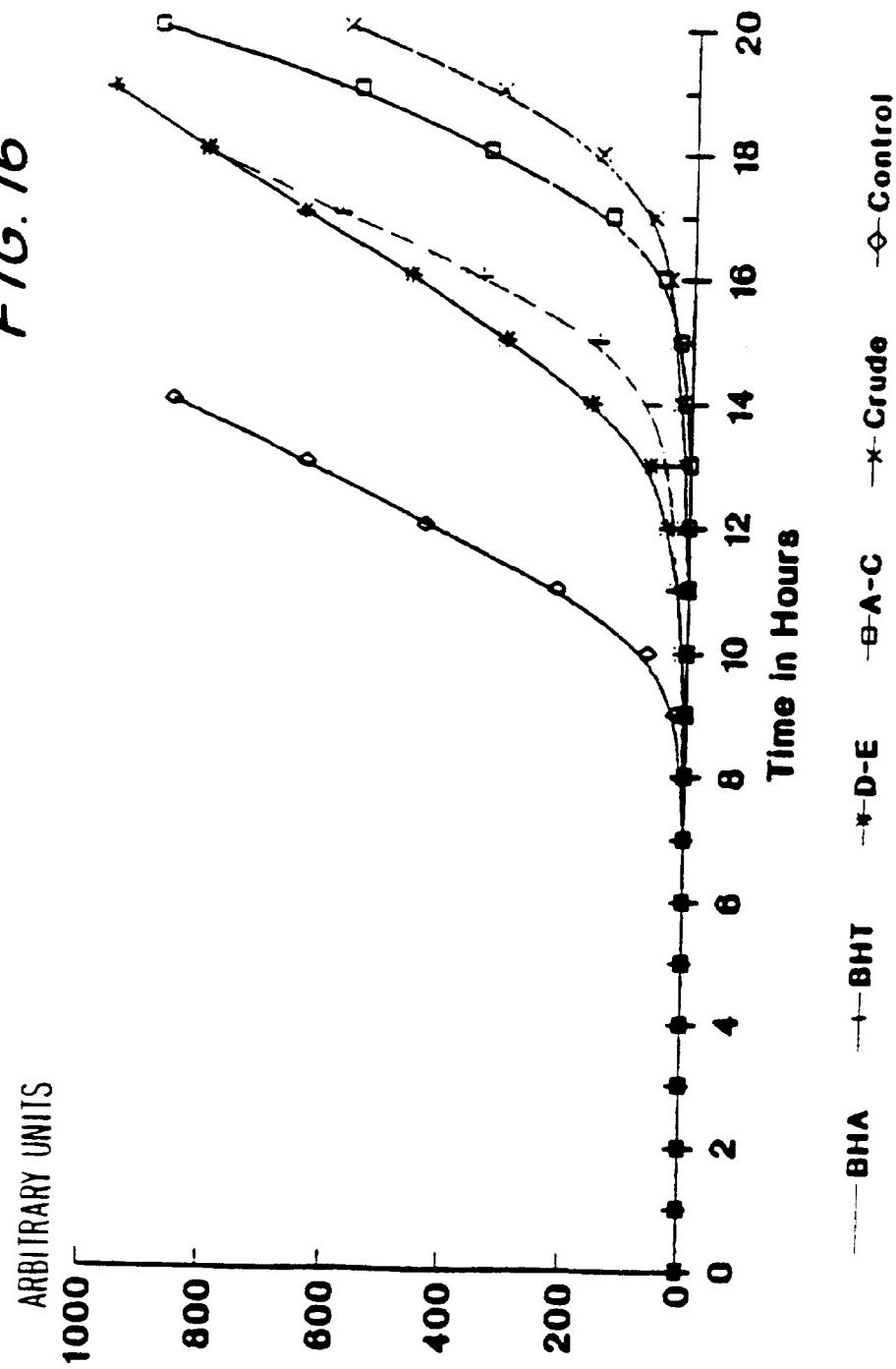
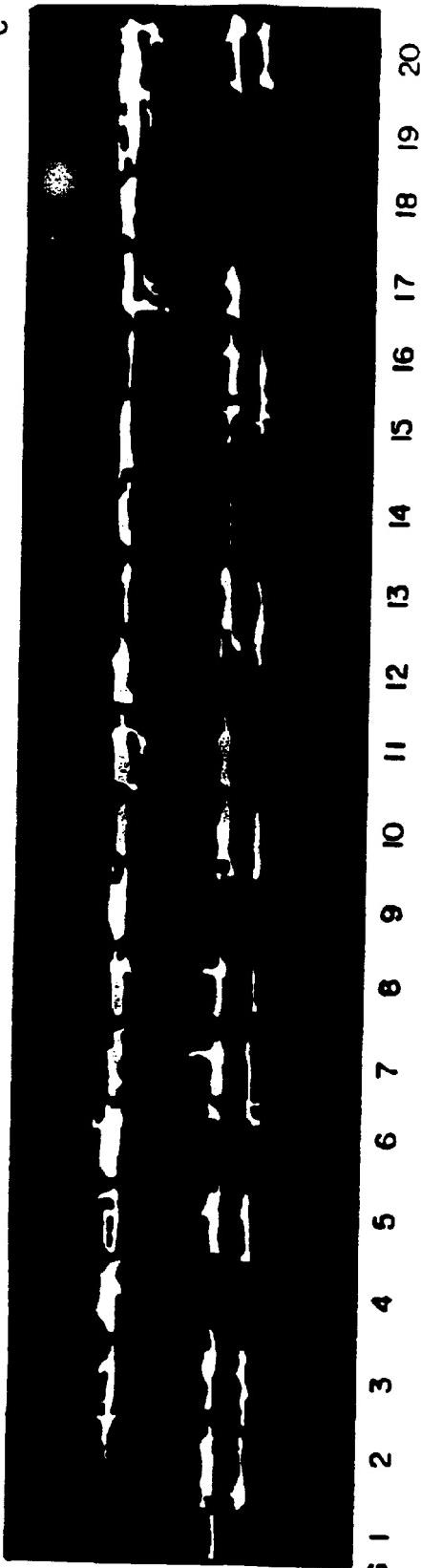
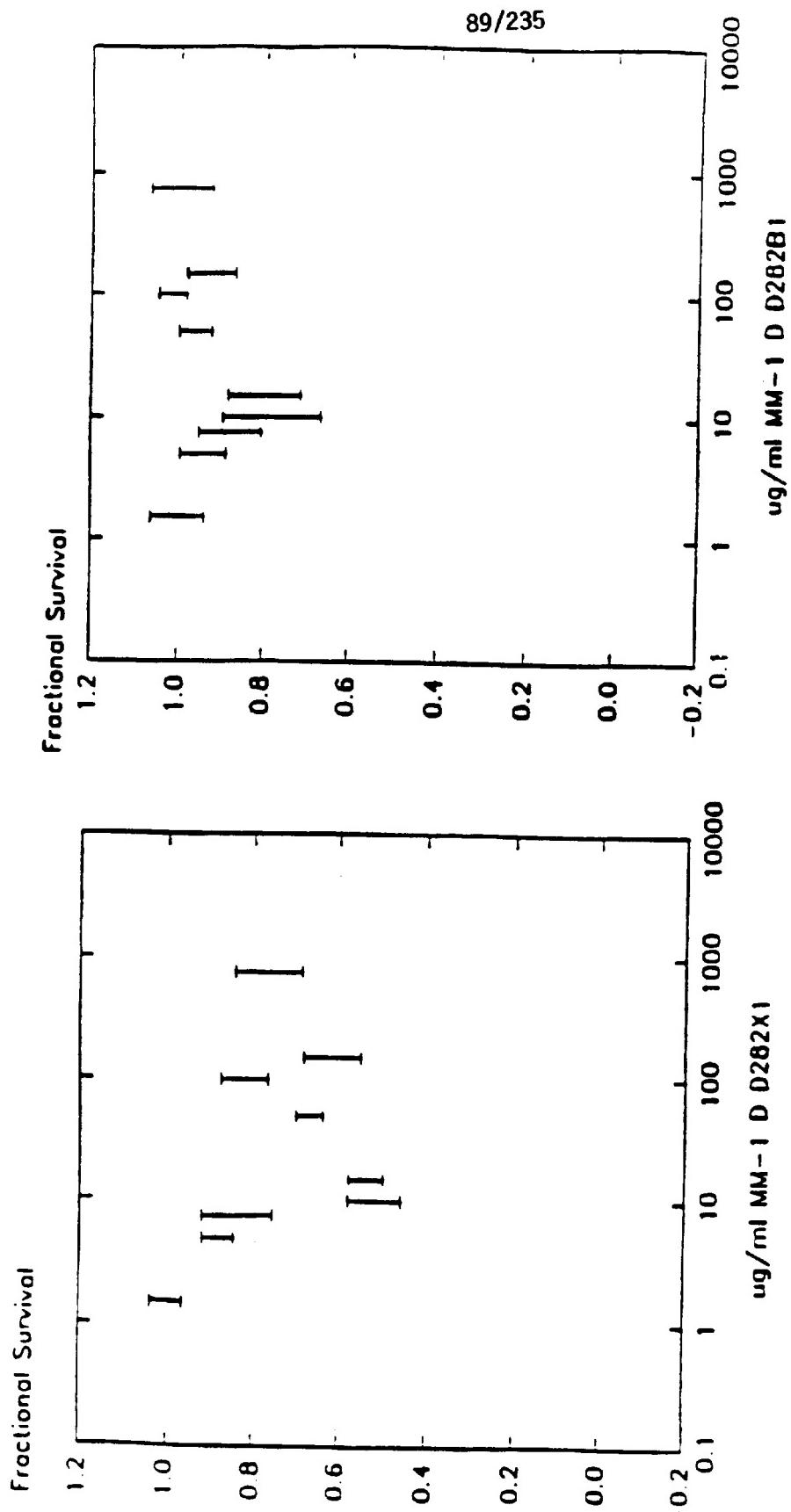


FIG. 17

FIG. 1. Fractionation of the protein mixture obtained from the supernatant of the 10% sucrose gradient.

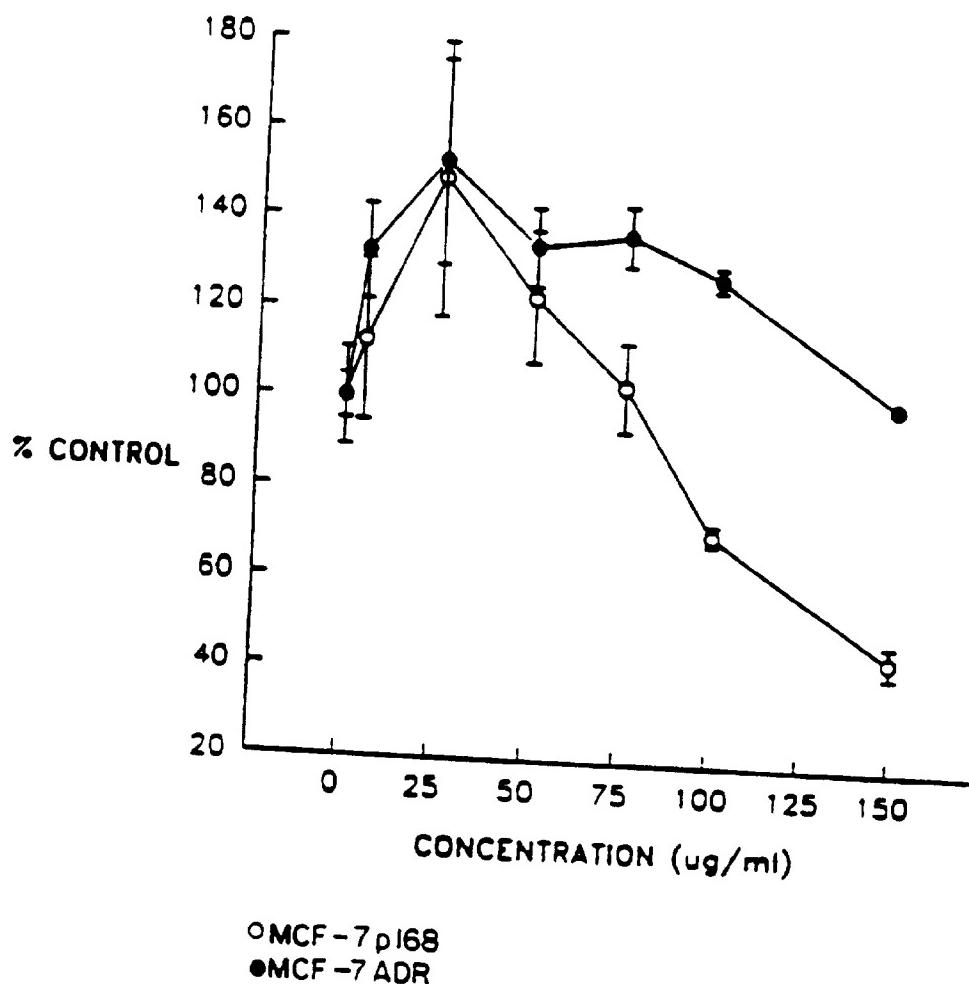


LANE 1 CONTAINS 0.5 μ g OF MARKER (M) MONOMER-LENGTH KINETOPLAST DNA CIRCLES.
LANES 2 AND 20 CONTAIN KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE PRESENCE OF 4% DMSO, BUT IN THE ABSENCE OF ANY COCOA PROCYANIDINS (CONTROL-C).
LANES 3 AND 4 CONTAIN KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE PRESENCE OF 0.5 AND 5.0 μ g/ml COCOA PROCYANIDIN FRACTION A.
LANES 5 AND 6 CONTAIN KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE PRESENCE OF 0.5 AND 5.0 μ g/ml COCOA PROCYANIDIN FRACTION B.
LANES 7, 8, 9, 13, 14 AND 15 ARE REPLICATES OF KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE PRESENCE OF 0.05, 0.5 AND 5.0 μ g/ml COCOA PROCYANIDIN FRACTION D.
LANES 10, 11, 12, 16, 17, AND 18 ARE REPLICATES OF KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE PRESENCE OF 0.05, 0.5 AND 5.0 μ g/ml COCOA PROCYANIDIN FRACTION E.
LANE 19 IS A REPLICATE OF KINETOPLAST DNA THAT WAS INCUBATED WITH TOPOISOMERASE II IN THE PRESENCE OF 5.0 μ g/ml COCOA PROCYANIDIN FRACTION E.



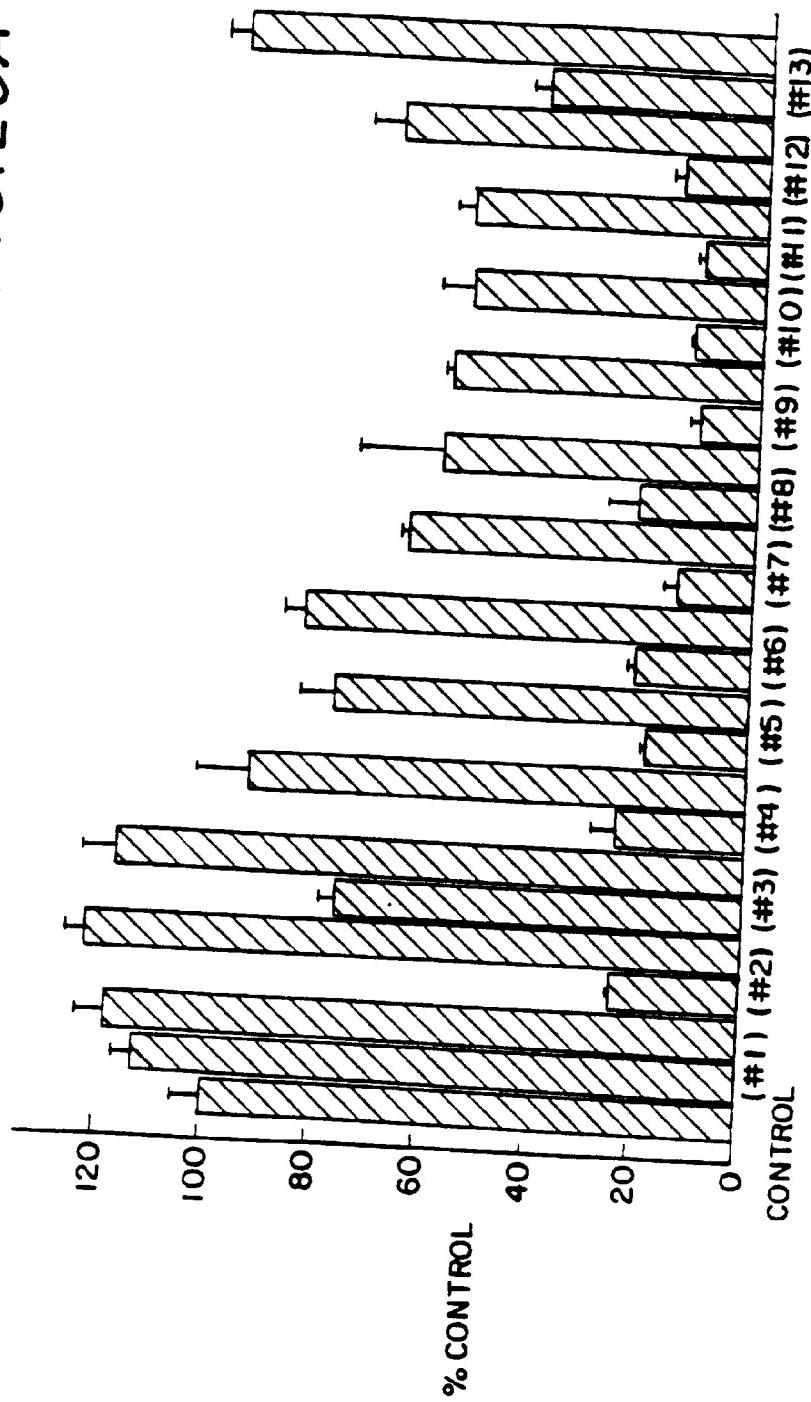
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FIG. 19



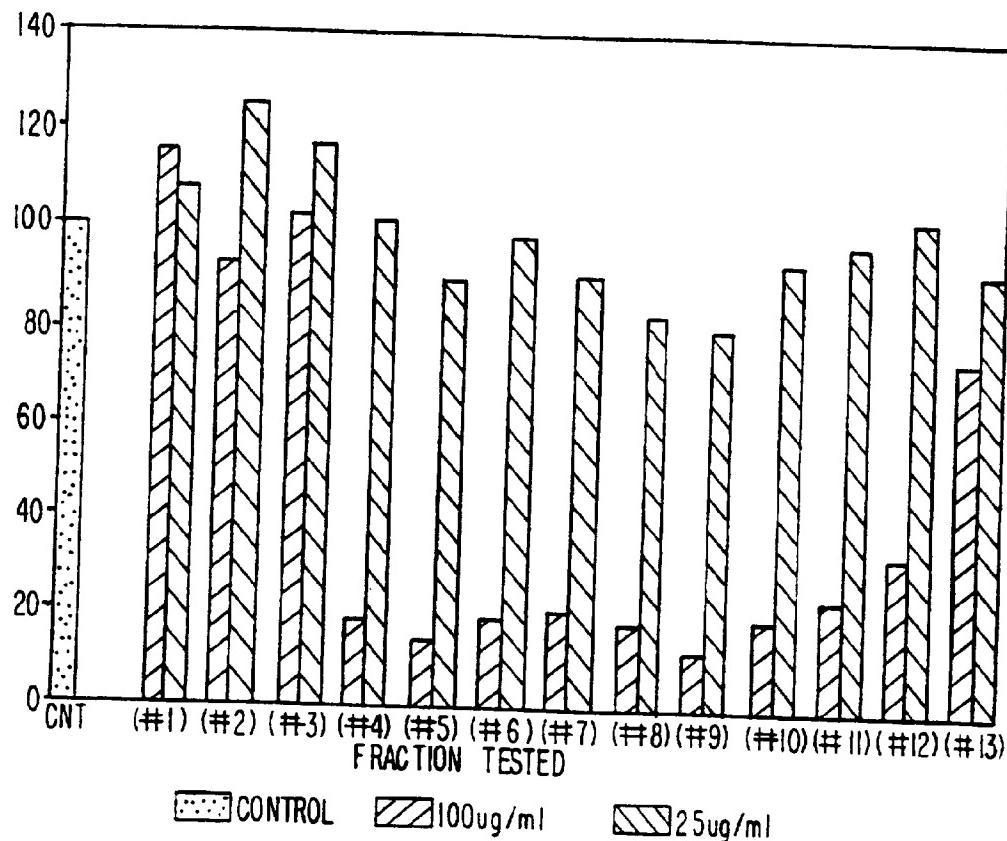
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FIG. 20A



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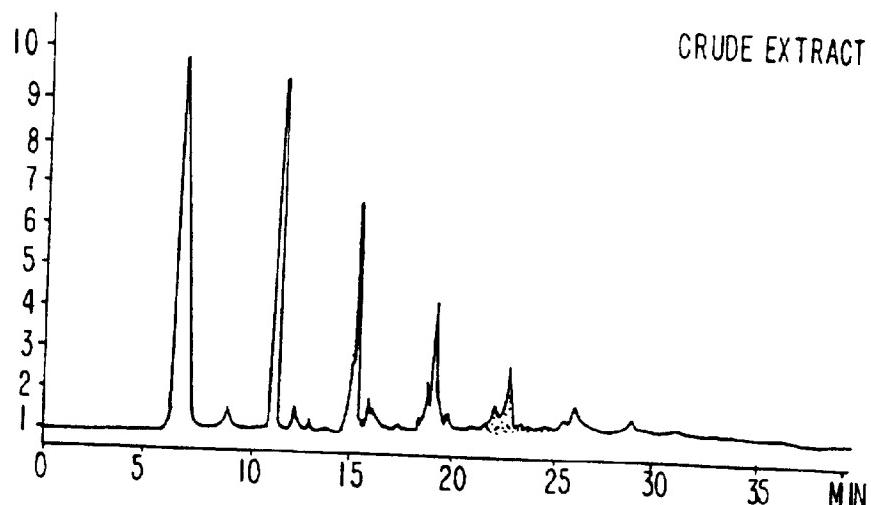
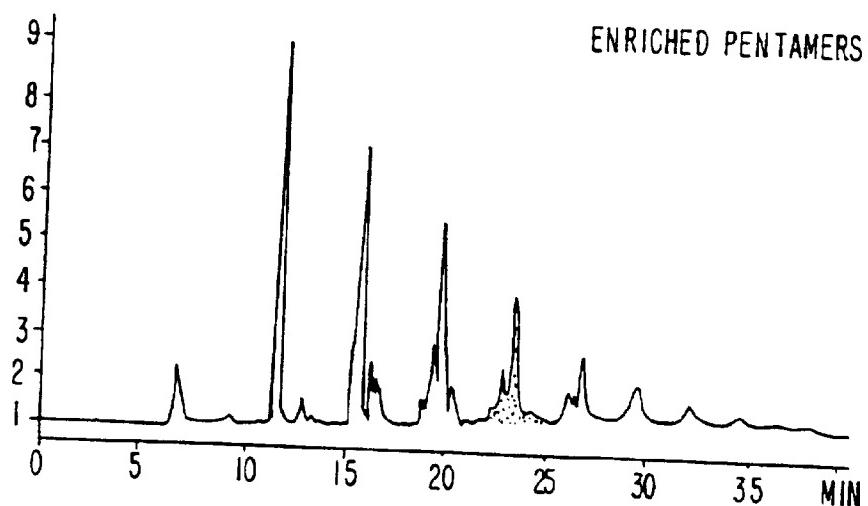
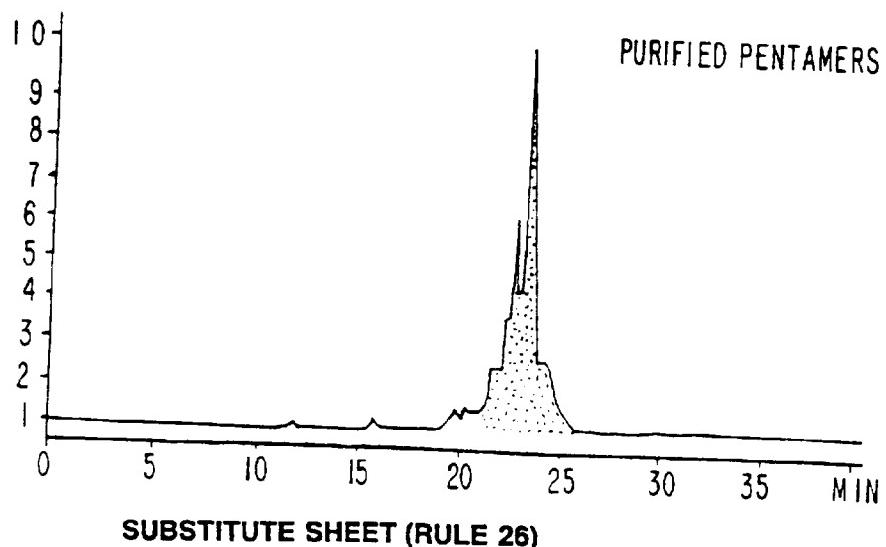
FIG. 20B

FRACTION NUMBEROLIGOMERIC UNIT

#1	DIMERS
#2	TRIMERS
#3	TETRAMERS
#4	PENTAMERS
#5	HEXAMERS
#6	HEPTAMERS
#7	OCTAMERS
#8	NONAMERS
#9	DECAMERS
#10	UNDECAMERS
#11	DODECAMERS
#12	HIGHER OLIGOMERS
#13	UNKNOWN

FIG. 21A

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**FIG. 21B****FIG. 21C**

SUBSTITUTE SHEET (RULE 26)

FIG. 22A

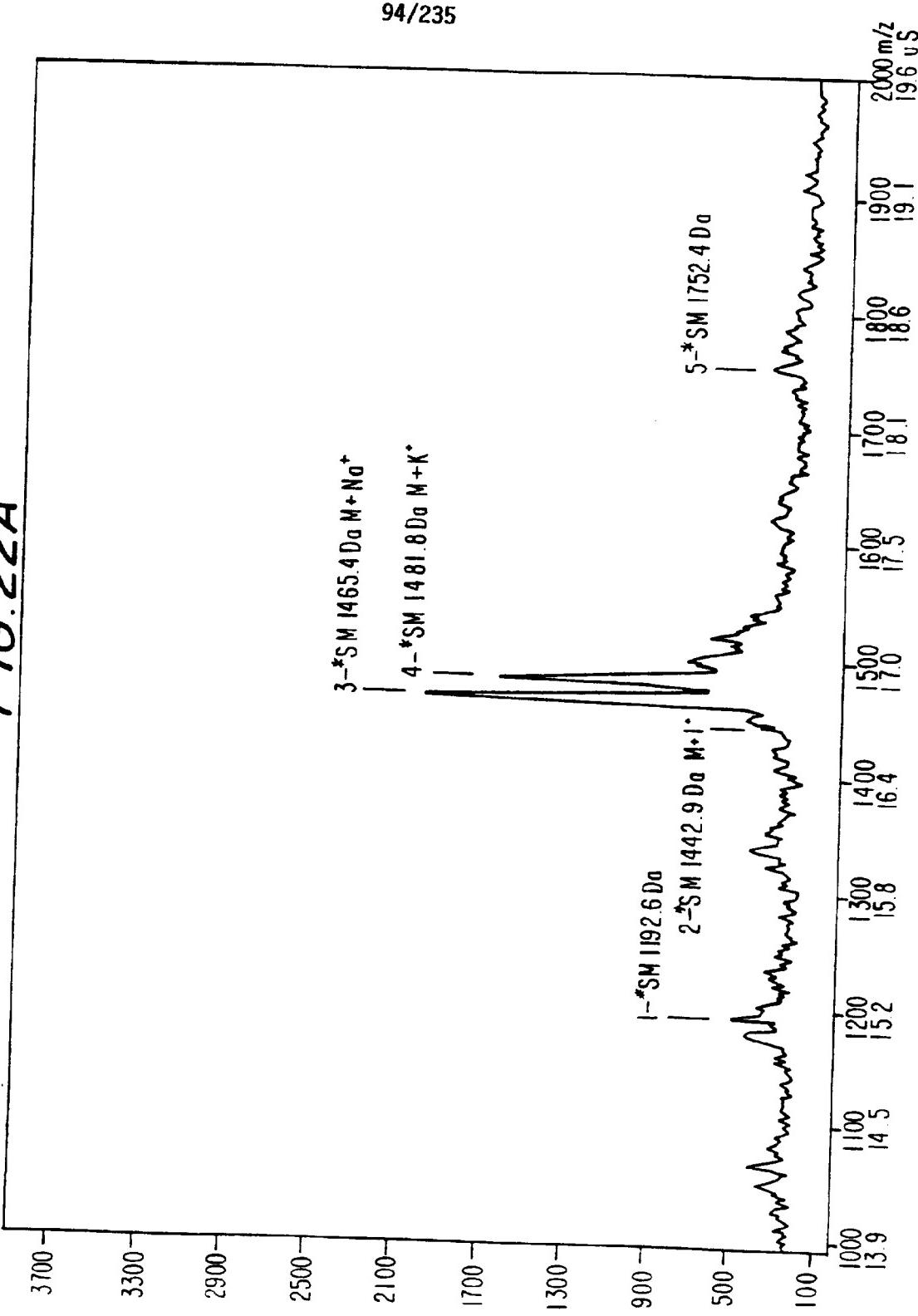
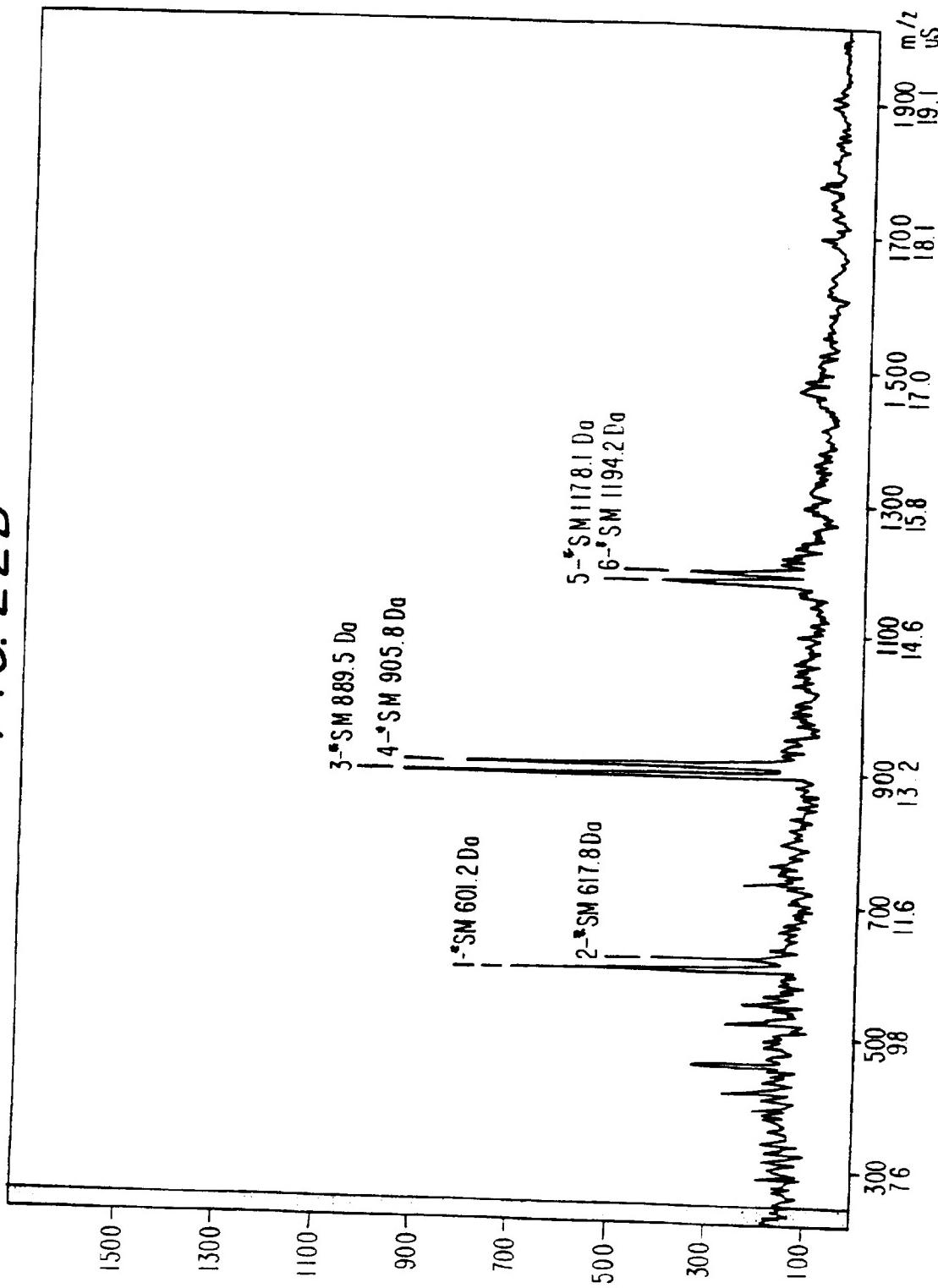
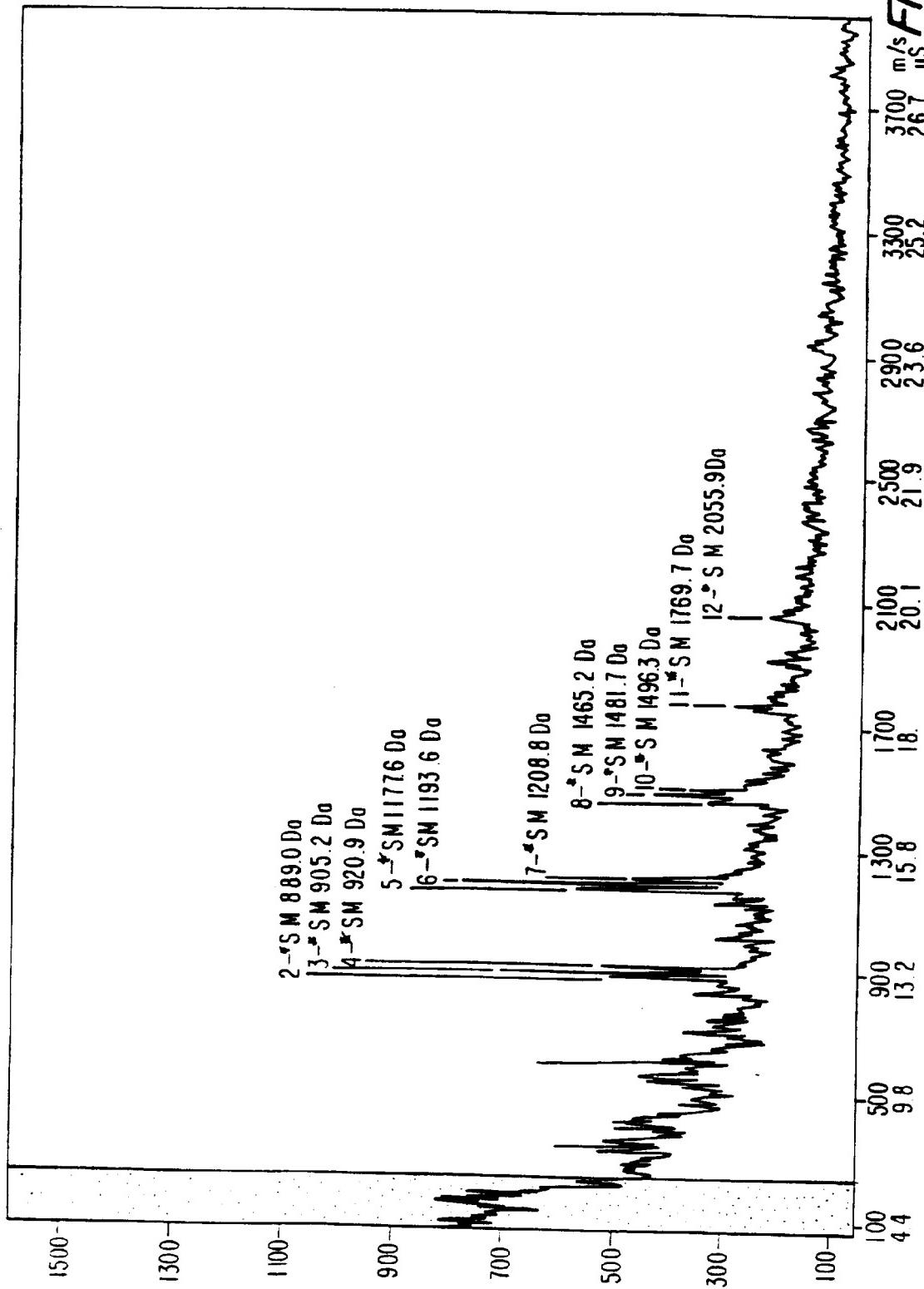


FIG. 22B

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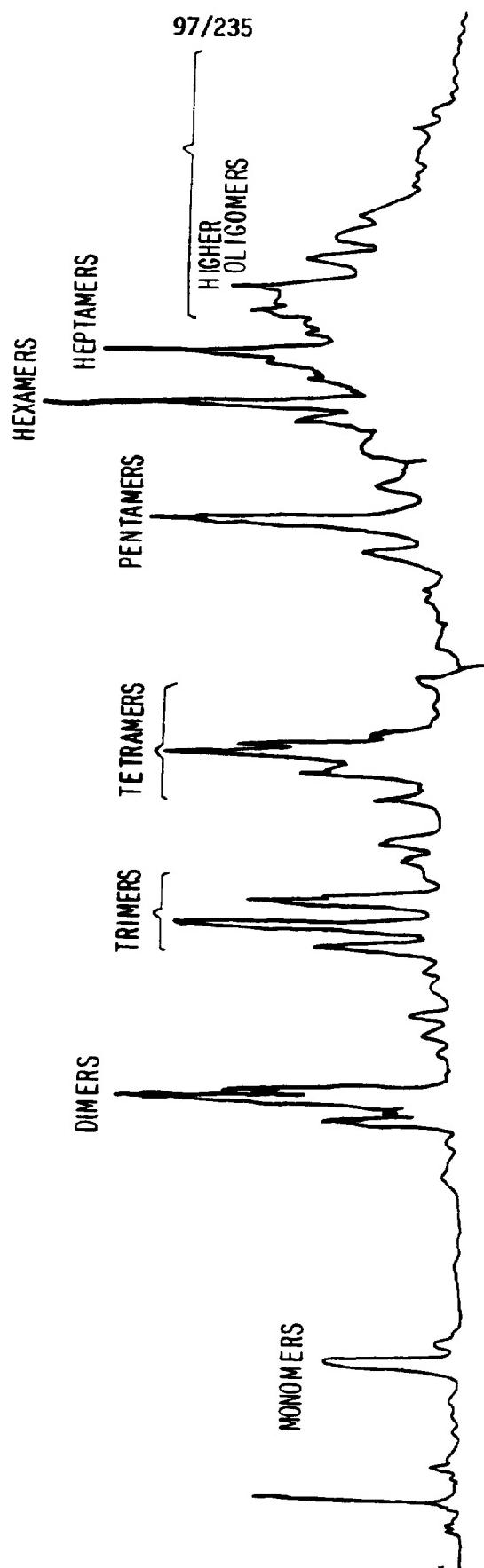
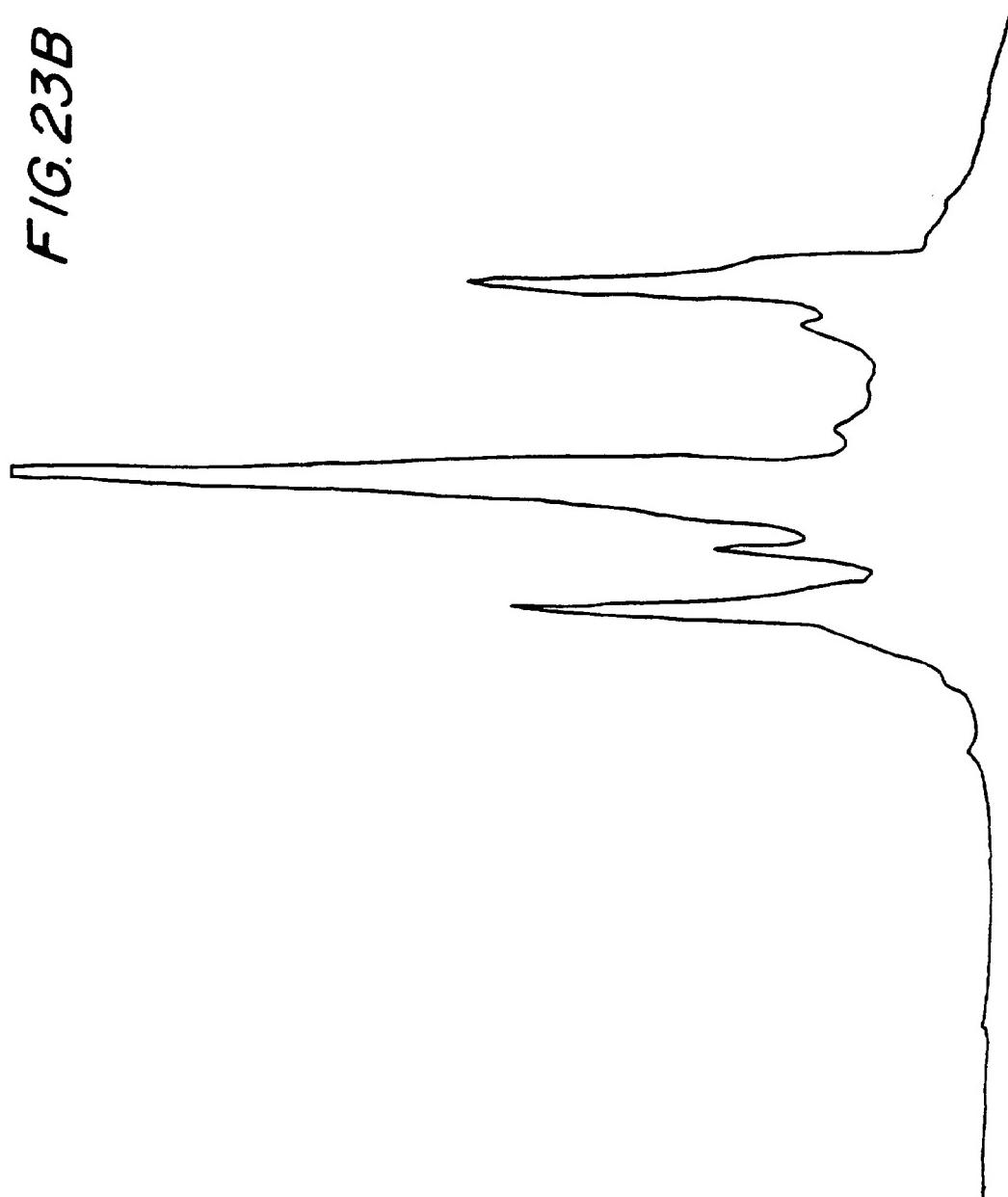


FIG. 23A

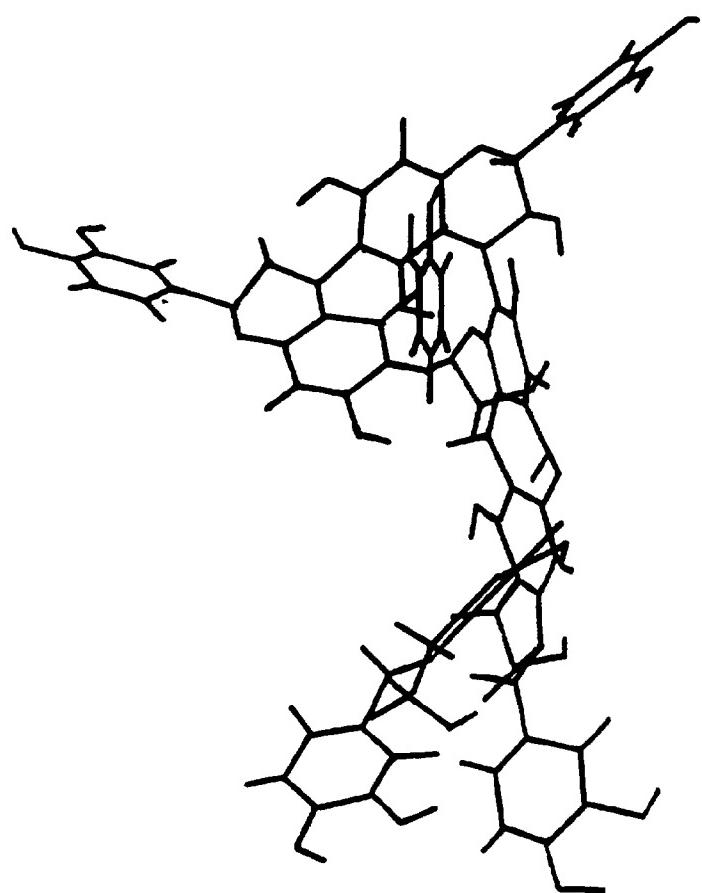
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FIG. 23B



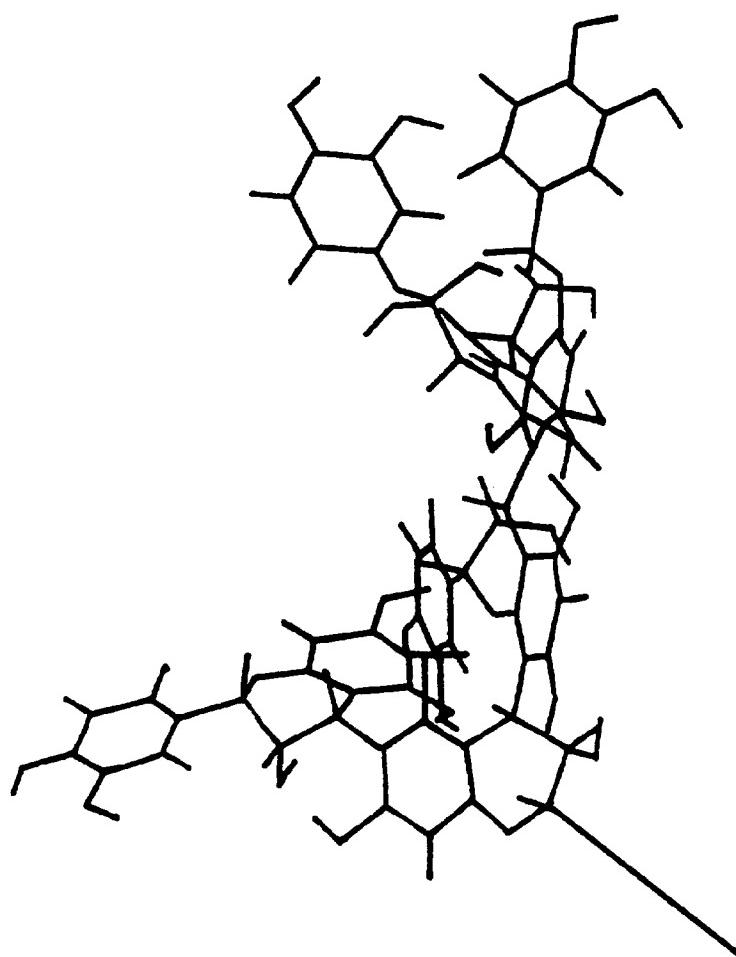
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FIG. 24A



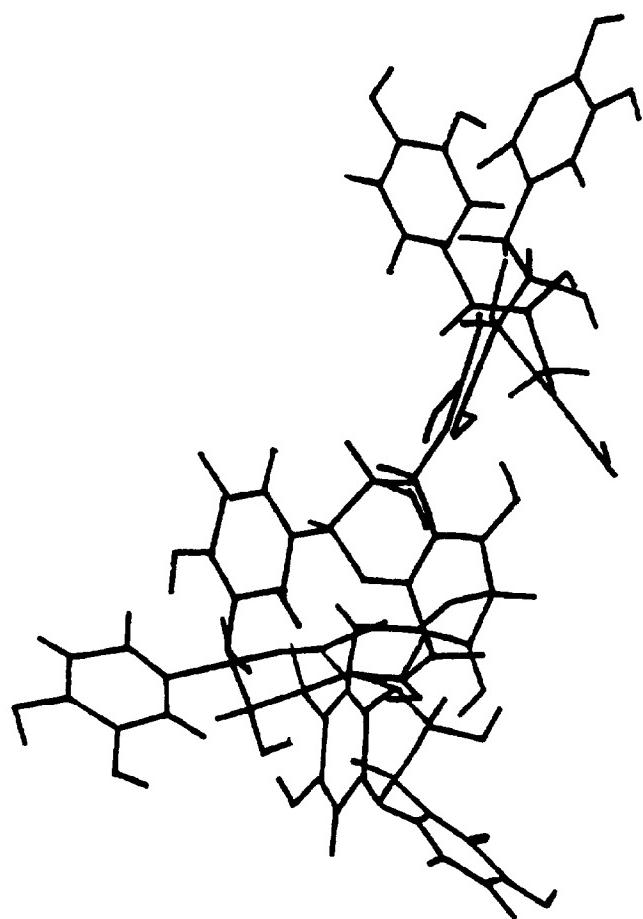
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FIG. 24B



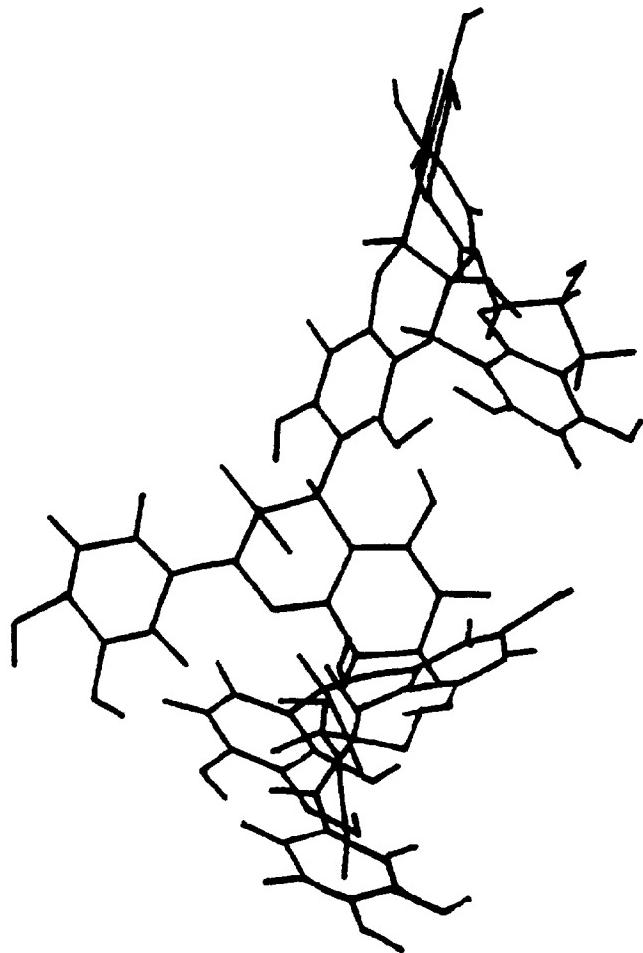
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FIG. 24C



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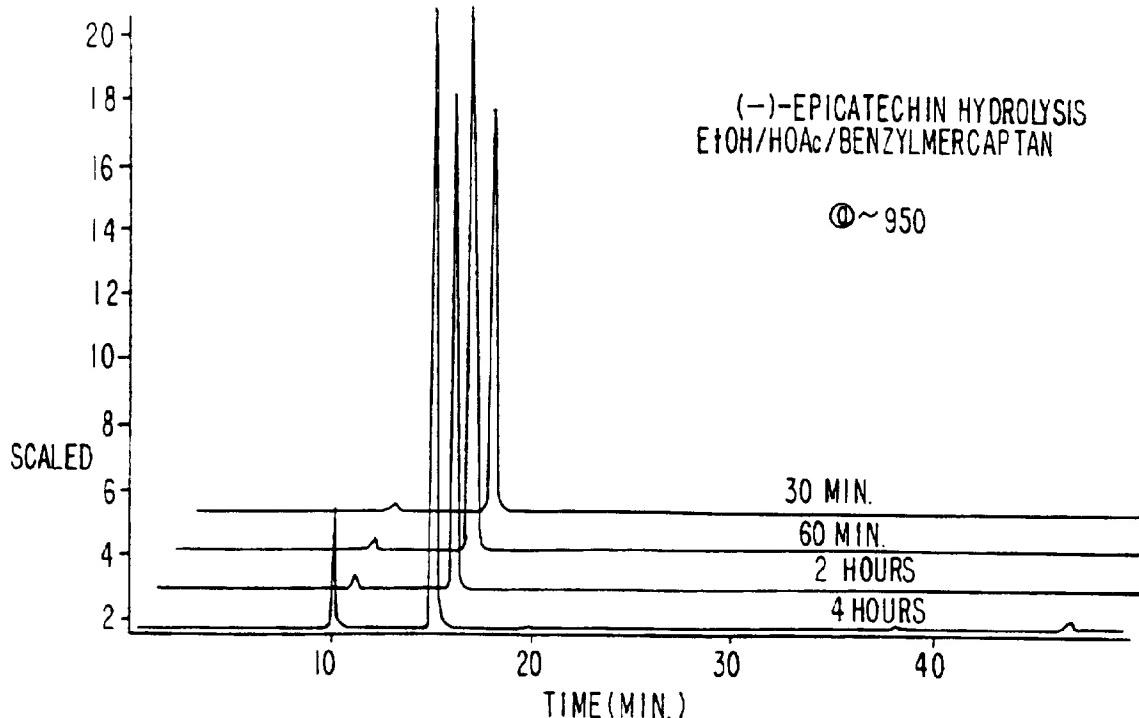
FIG. 24D



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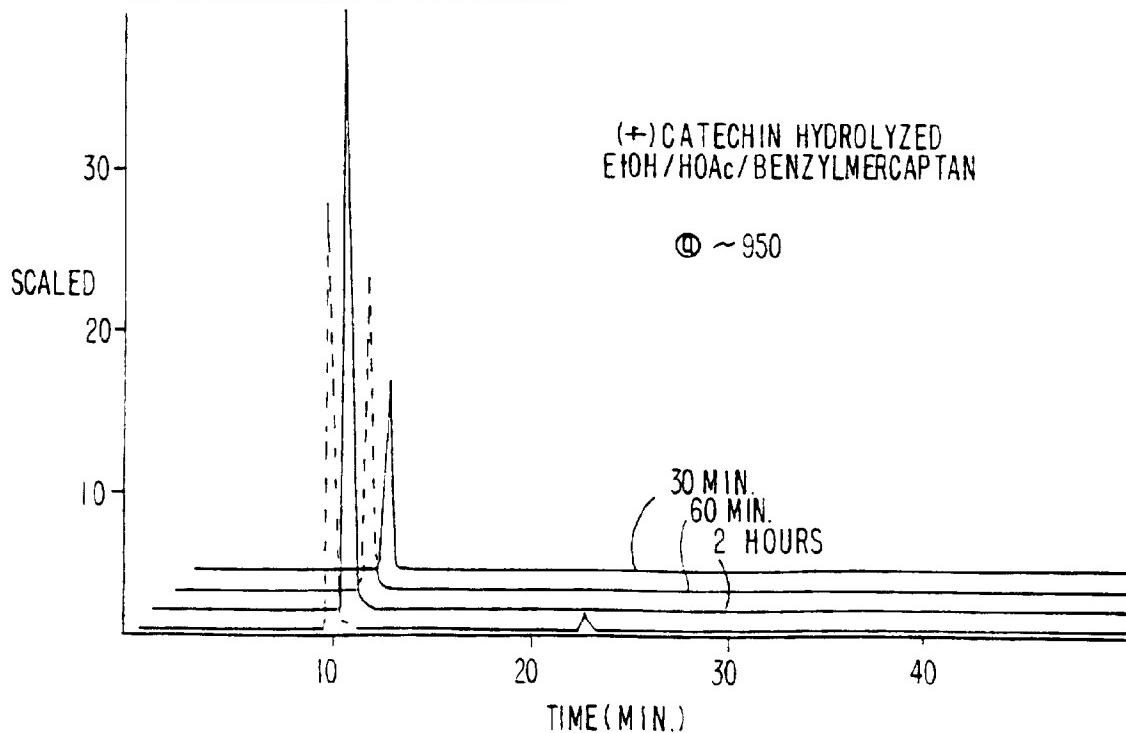
LC X FLUORESCENCE OF 6057_45A.D
 LC X FLUORESCENCE OF 6057_46A.D
 LC X FLUORESCENCE OF 6057_47A.D
 LC X FLUORESCENCE OF 6057_48A.D

FIG. 25A



LC X FLUORESCENCE OF 6057_49A.D
 LC X FLUORESCENCE OF 6057_50A.D
 LC X FLUORESCENCE OF 6057_51A.D
 LC X FLUORESCENCE OF 6057_52A.D

FIG. 25B



SUBSTITUTE SHEET (RULE 26)

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FIG. 25C

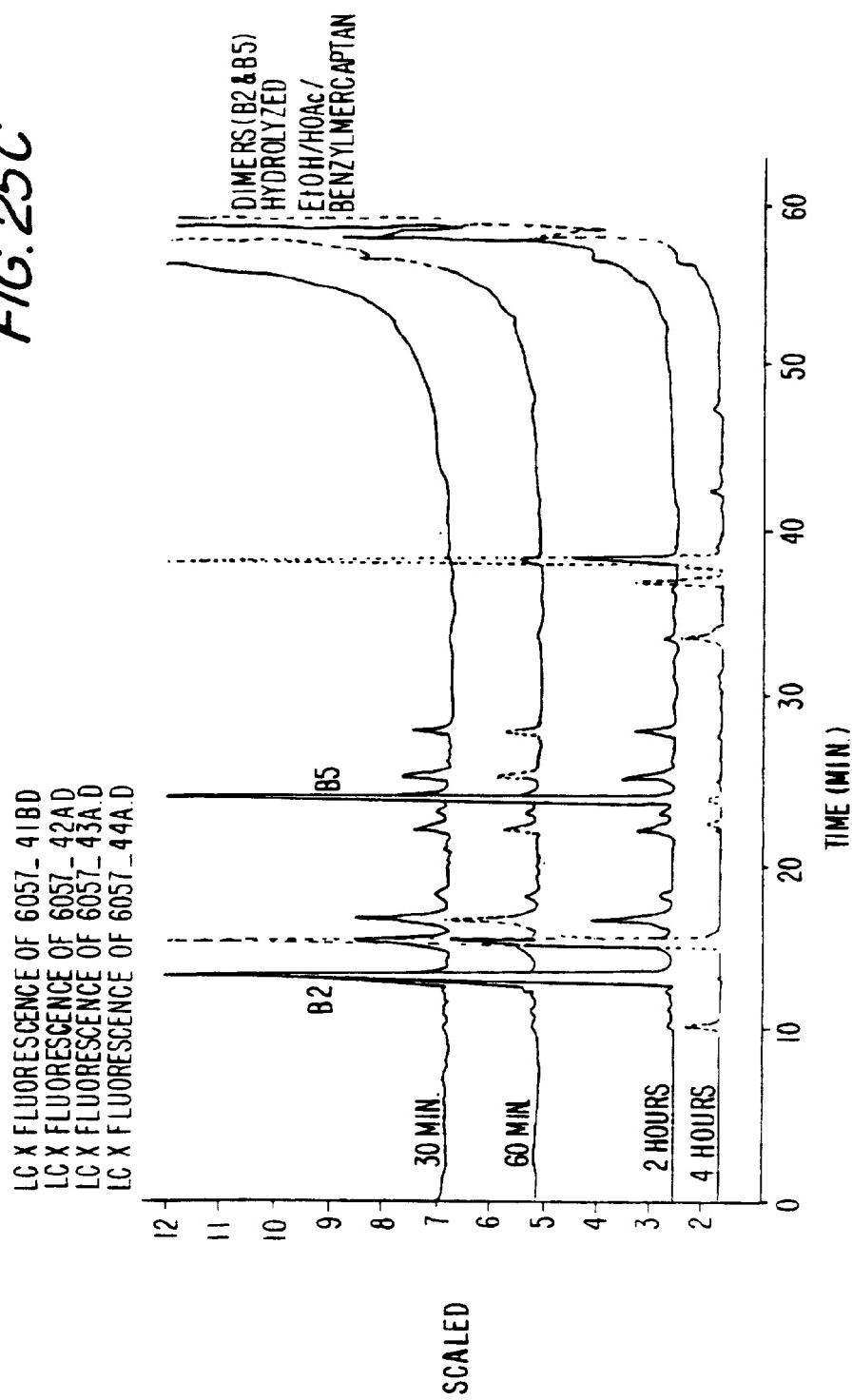
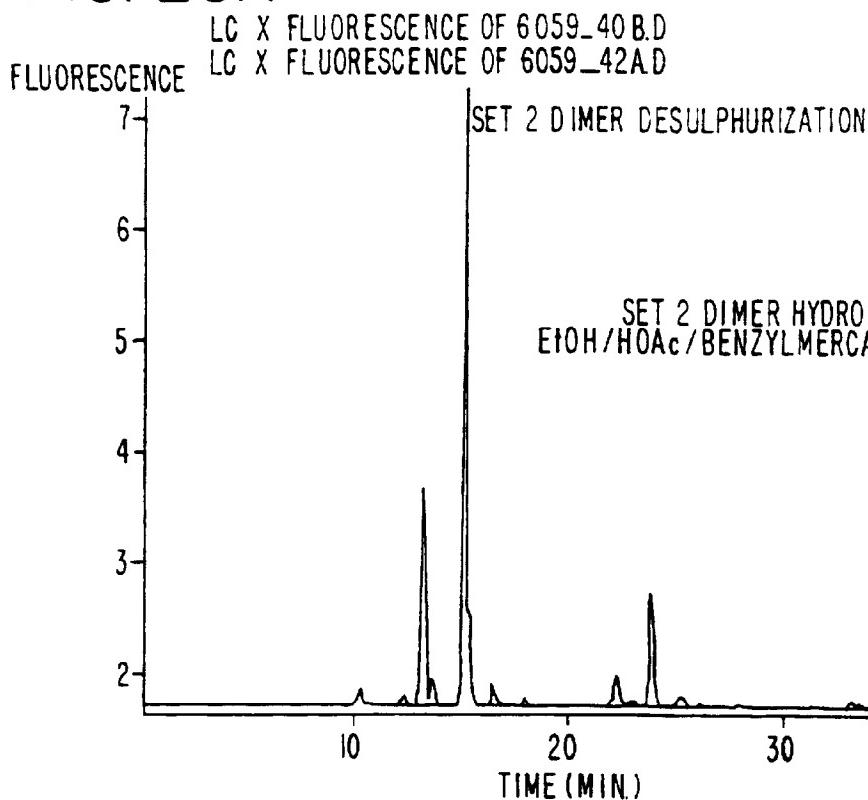
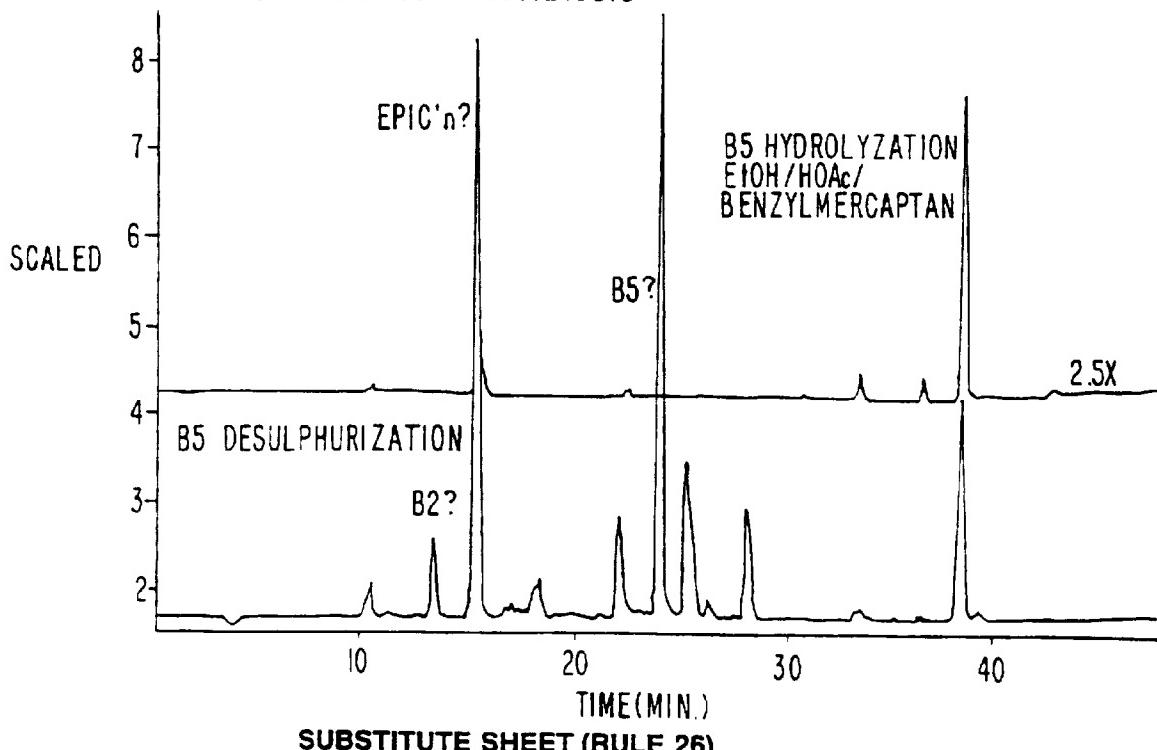


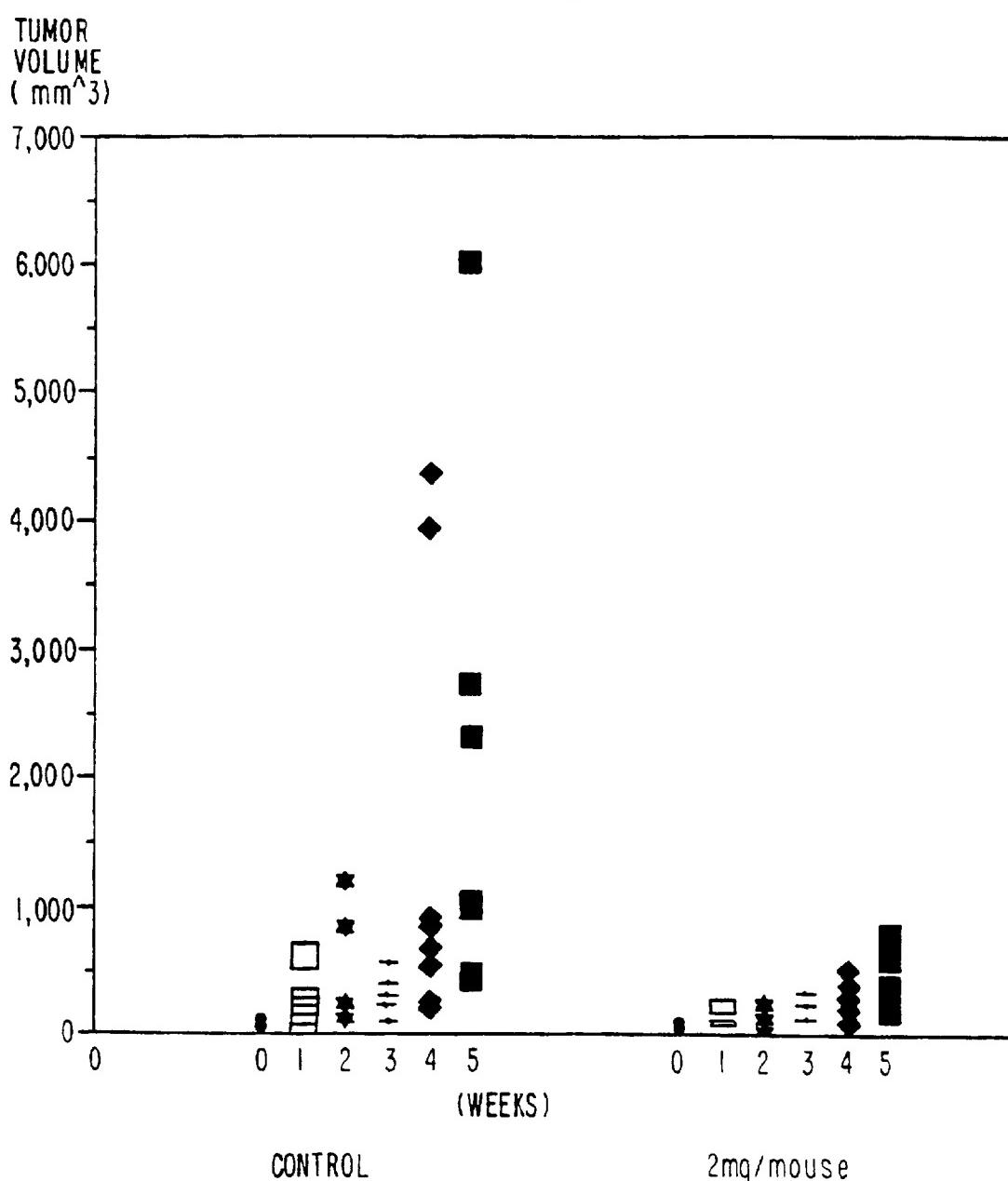
FIG. 26A

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**FIG. 26B**LC X FLUORESCENCE OF 6059_41 A.D
LC X FLUORESCENCE OF 6060_43 B.D

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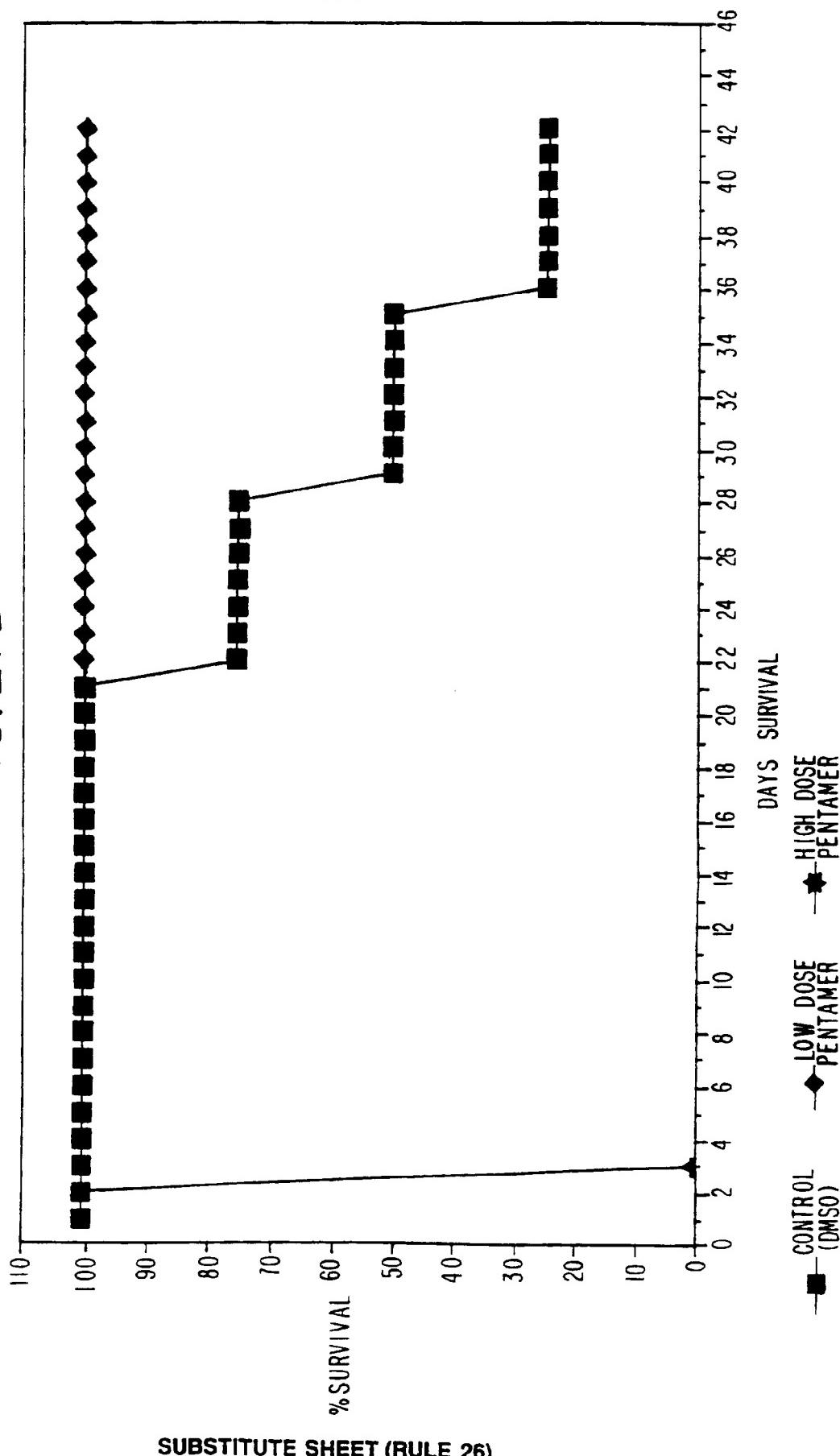
FIG. 27A



T VALUE = 2.68 P VALUE = 0.02

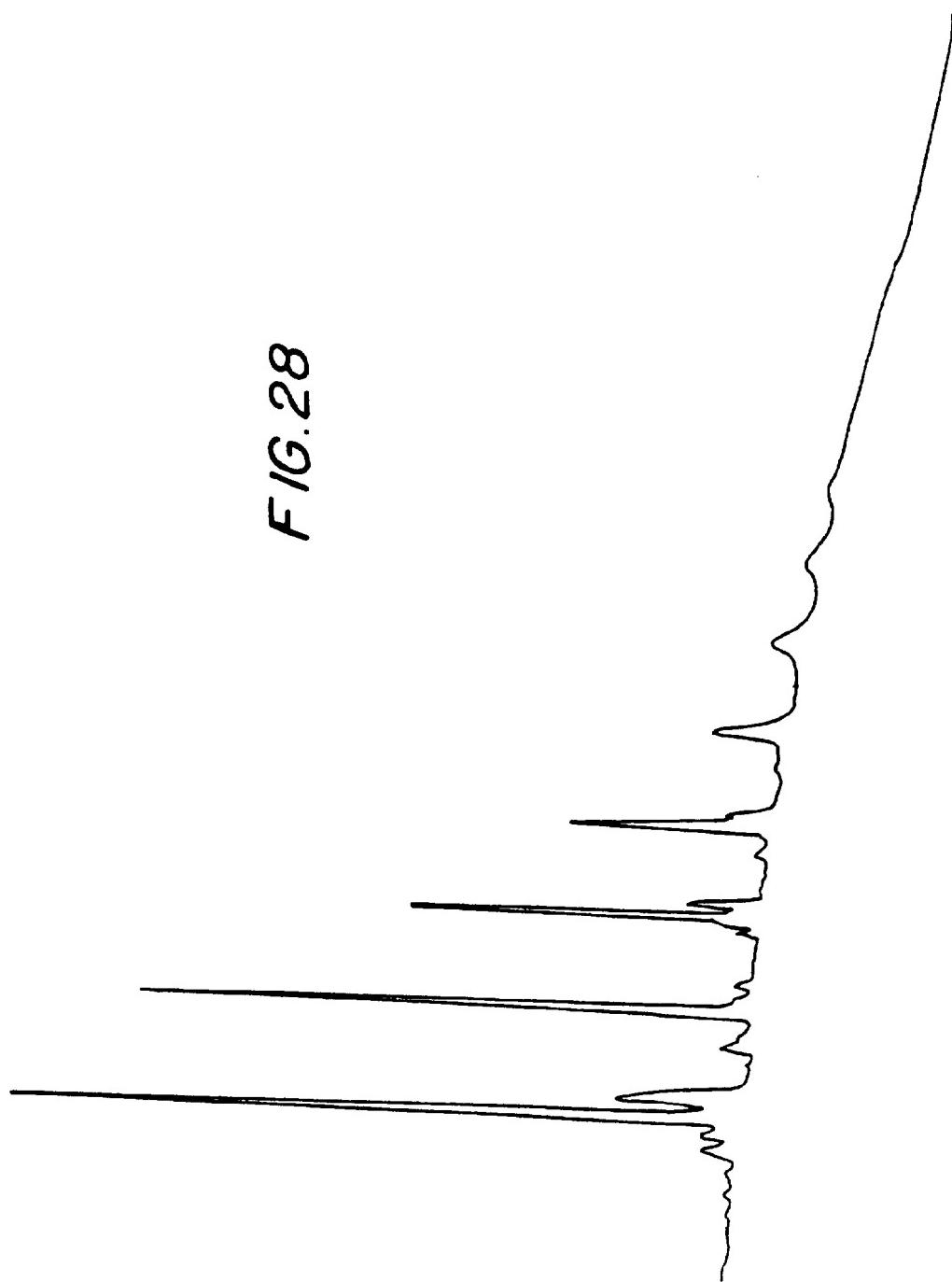
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FIG. 27B



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FIG. 28



SUBSTITUTE SHEET (RULE 26)

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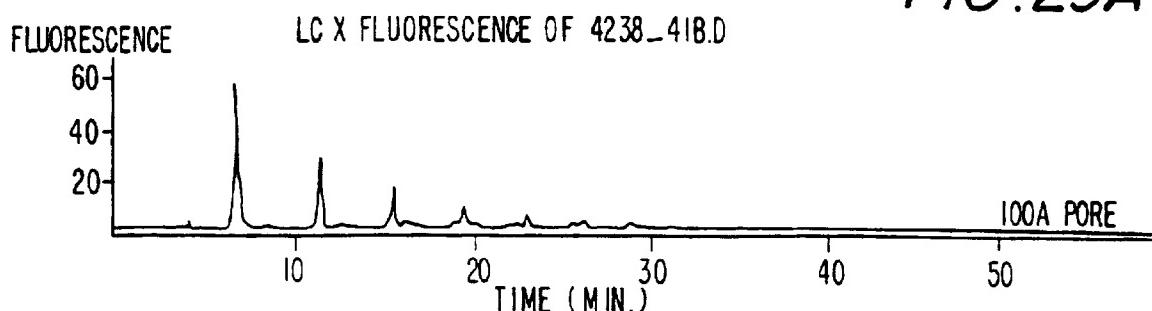
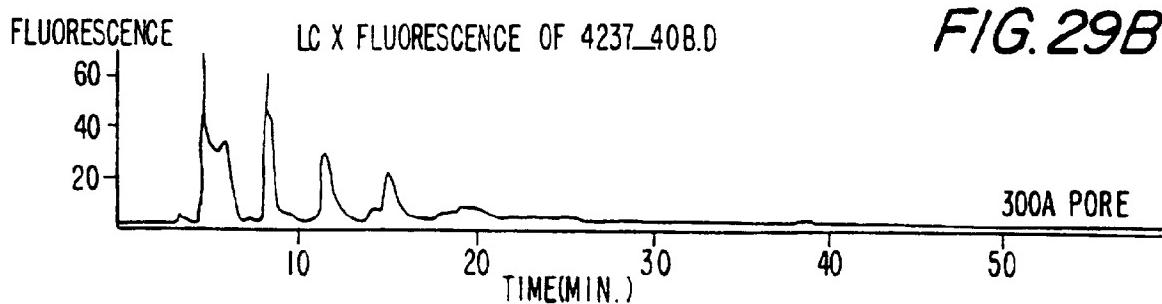
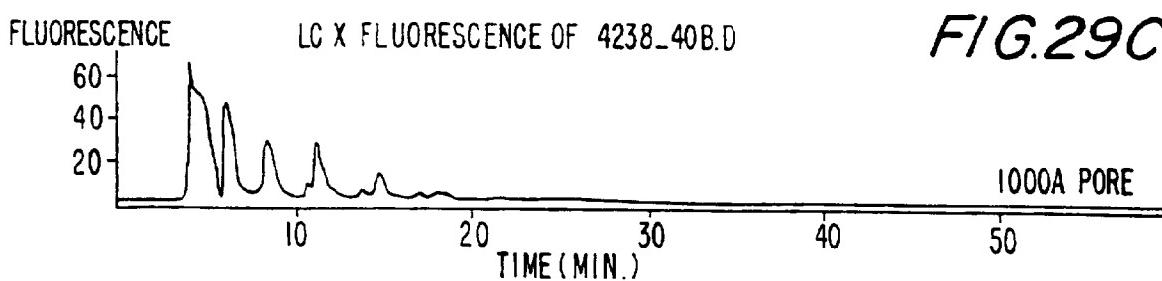
FIG. 29A*FIG. 29B**FIG. 29C*

FIG. 30A

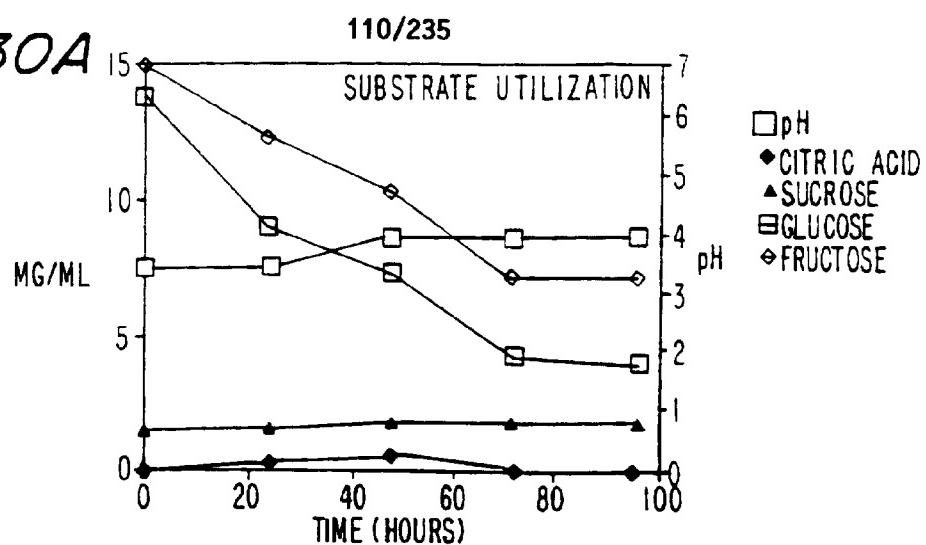


FIG. 30B

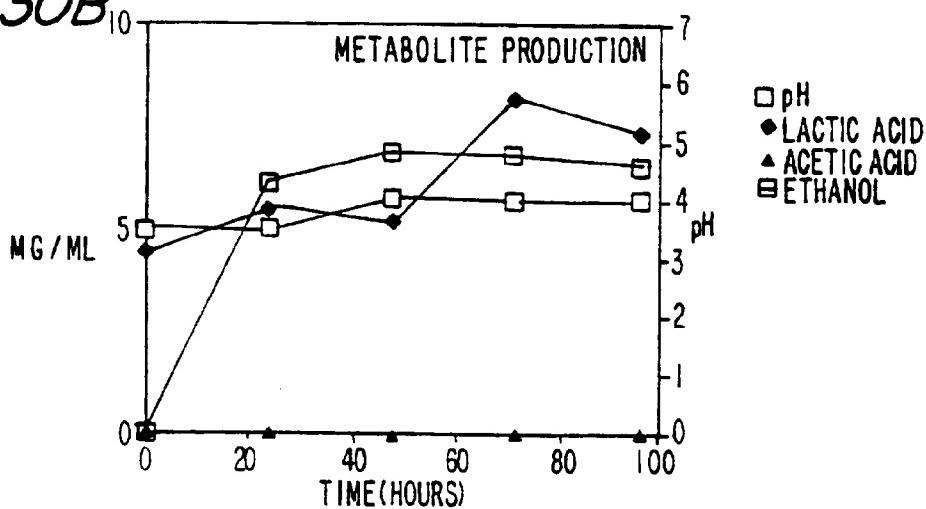
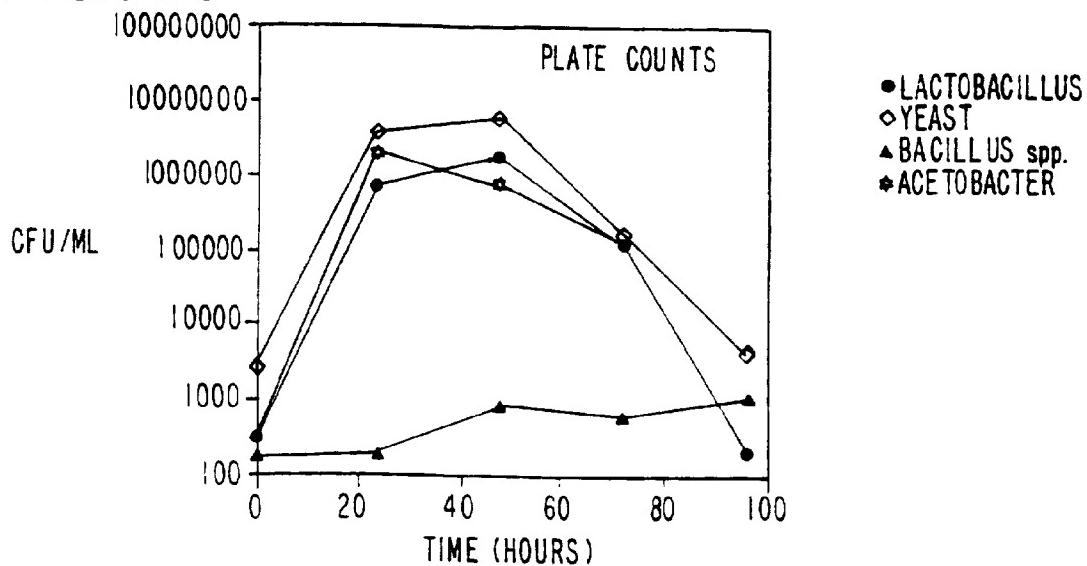
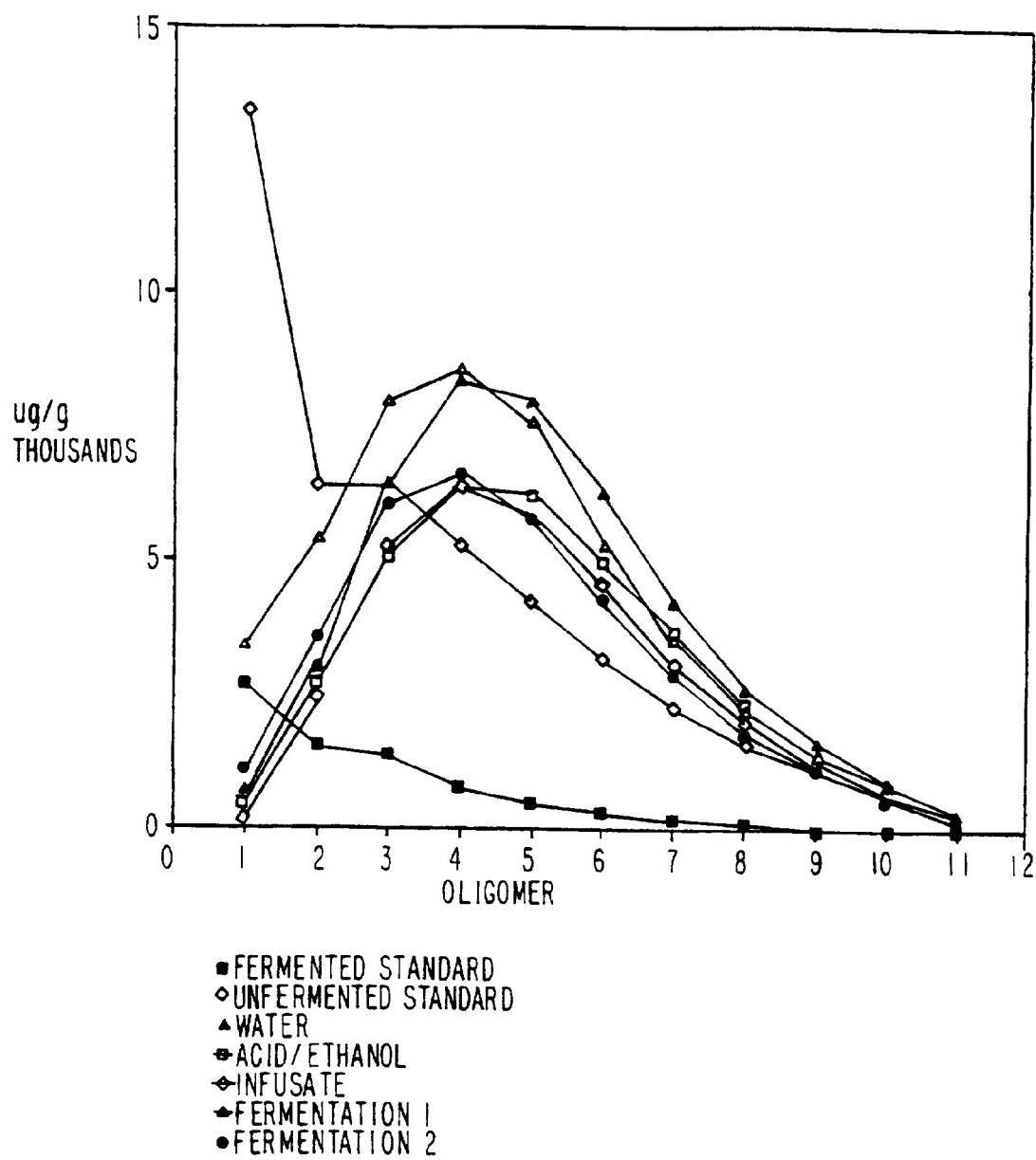


FIG. 30C



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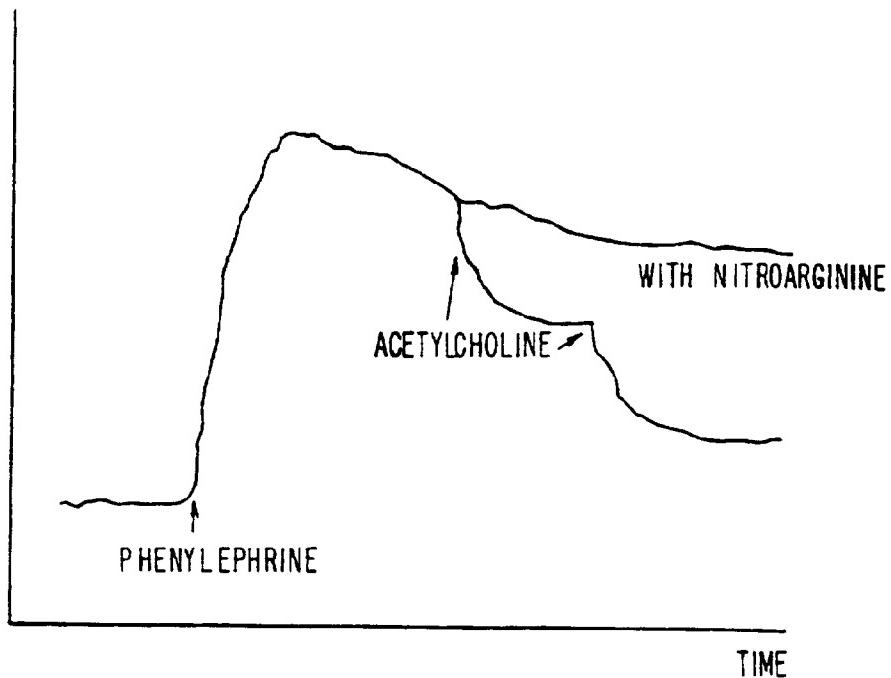
FIG. 30D



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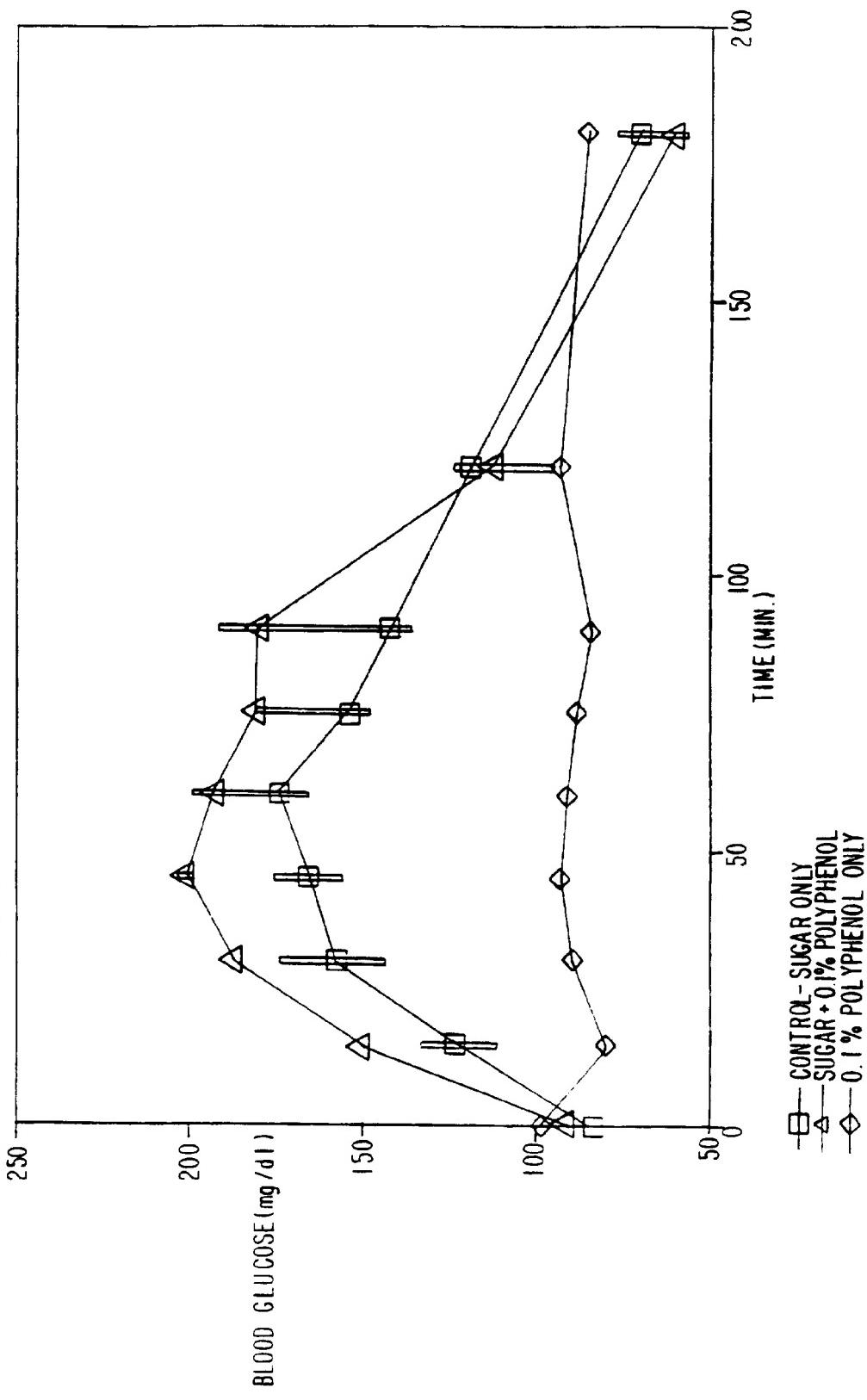
FIG.31

CONTRACTION OF ISOLATED AORTA



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FIG. 32



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FIG. 33A

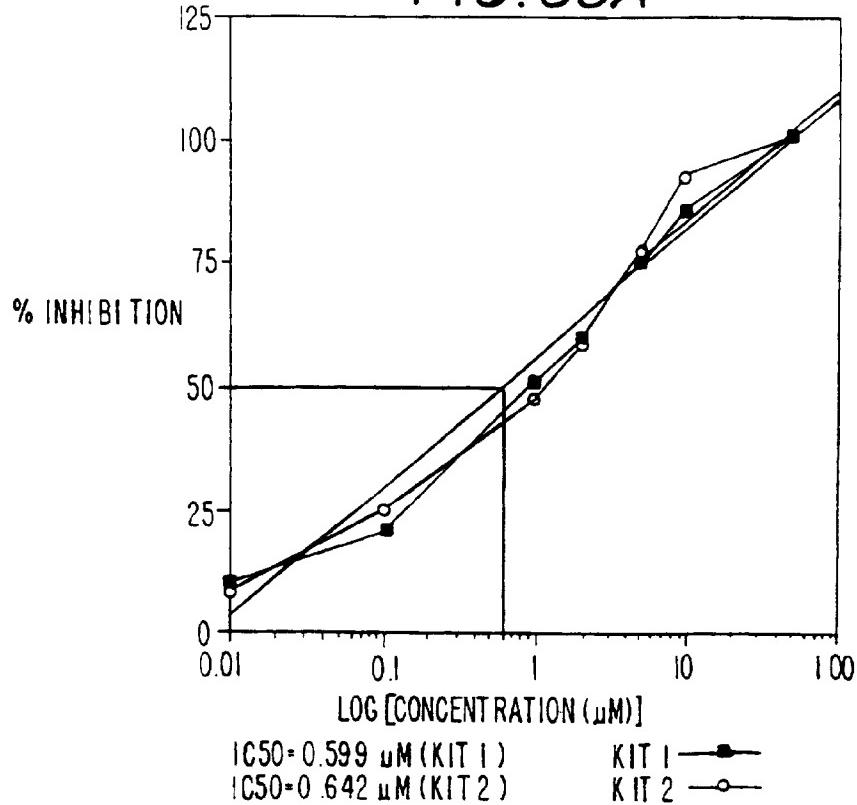
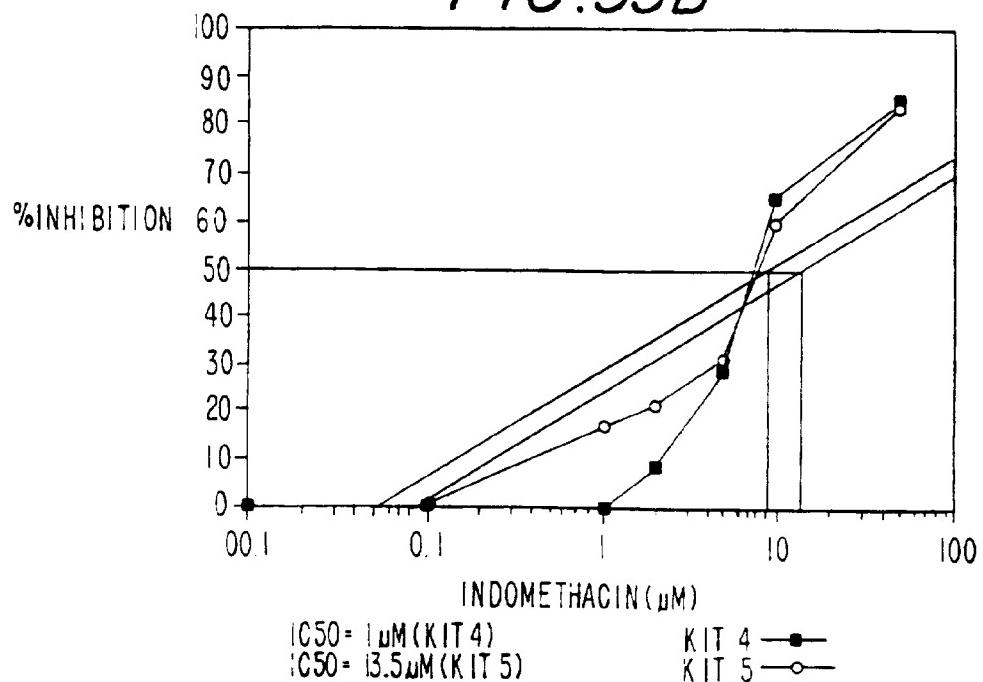
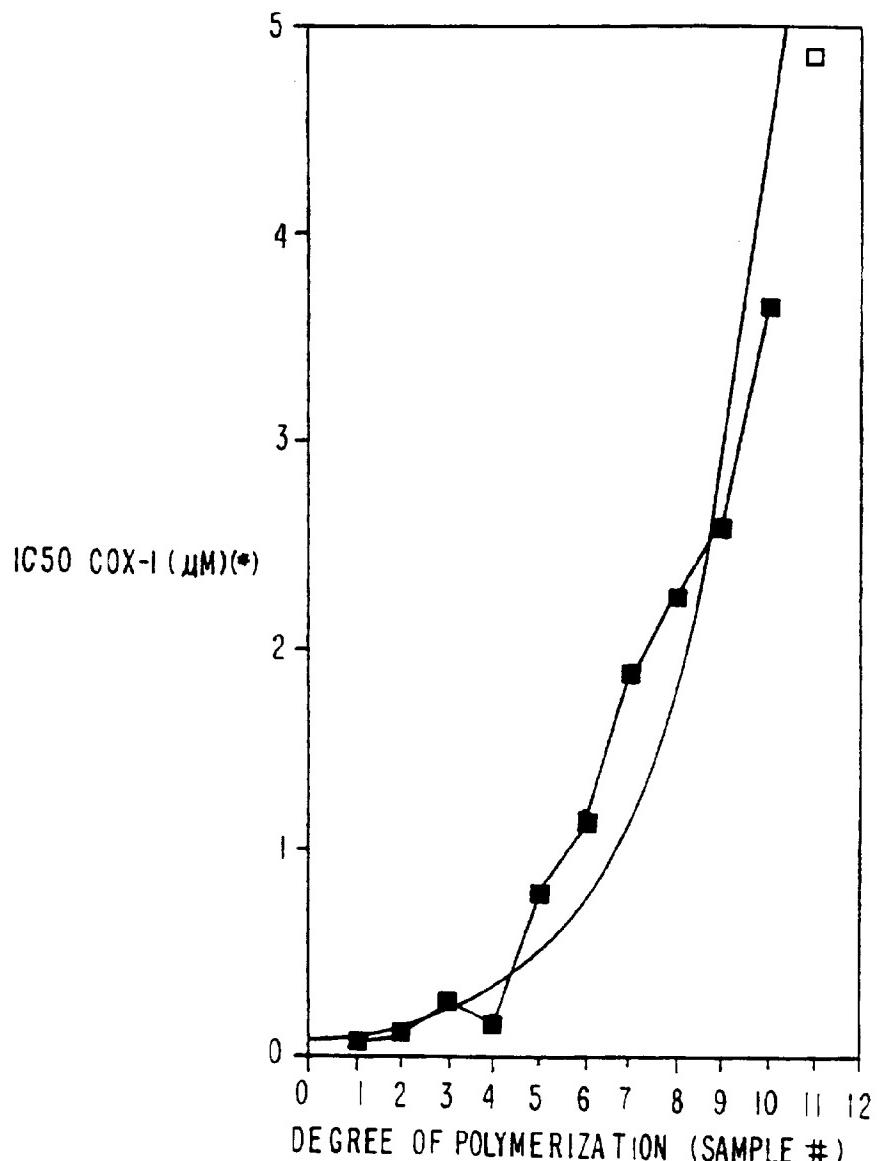


FIG. 33B



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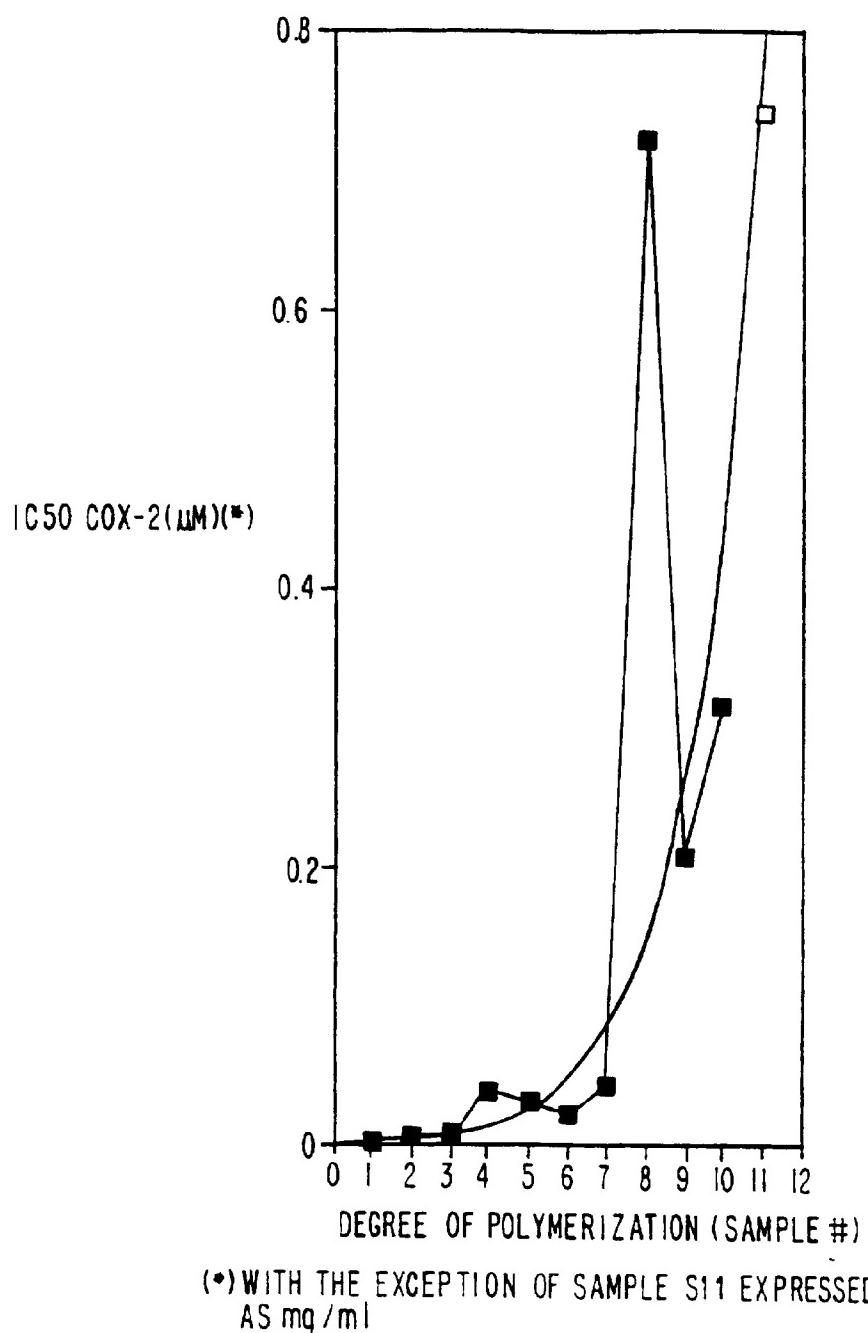
FIG. 34A



(*) WITH THE EXCEPTION OF SAMPLE S11 EXPRESSED AS mg/ml

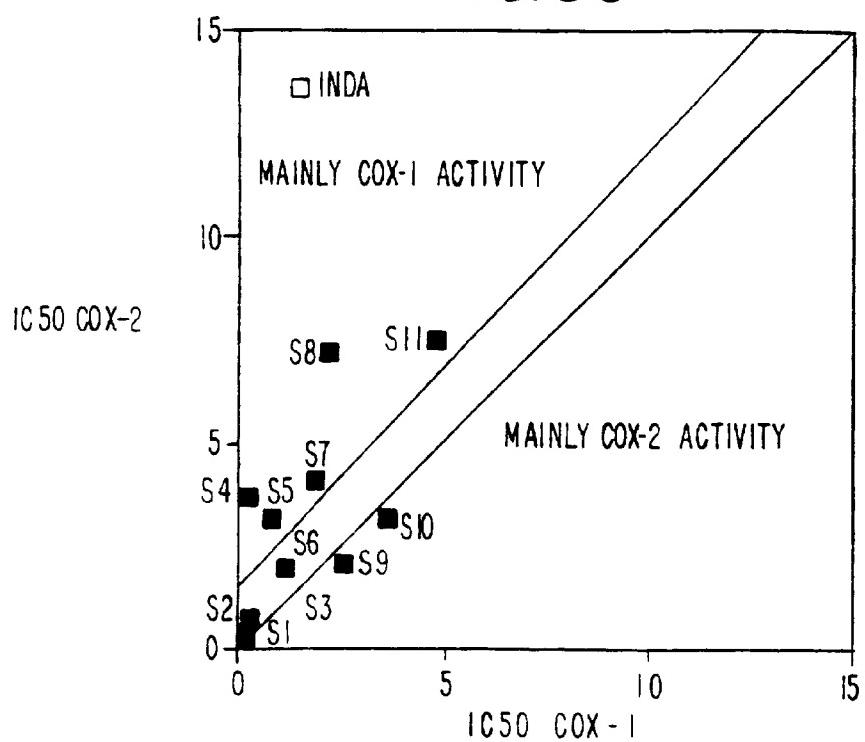
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FIG. 34B



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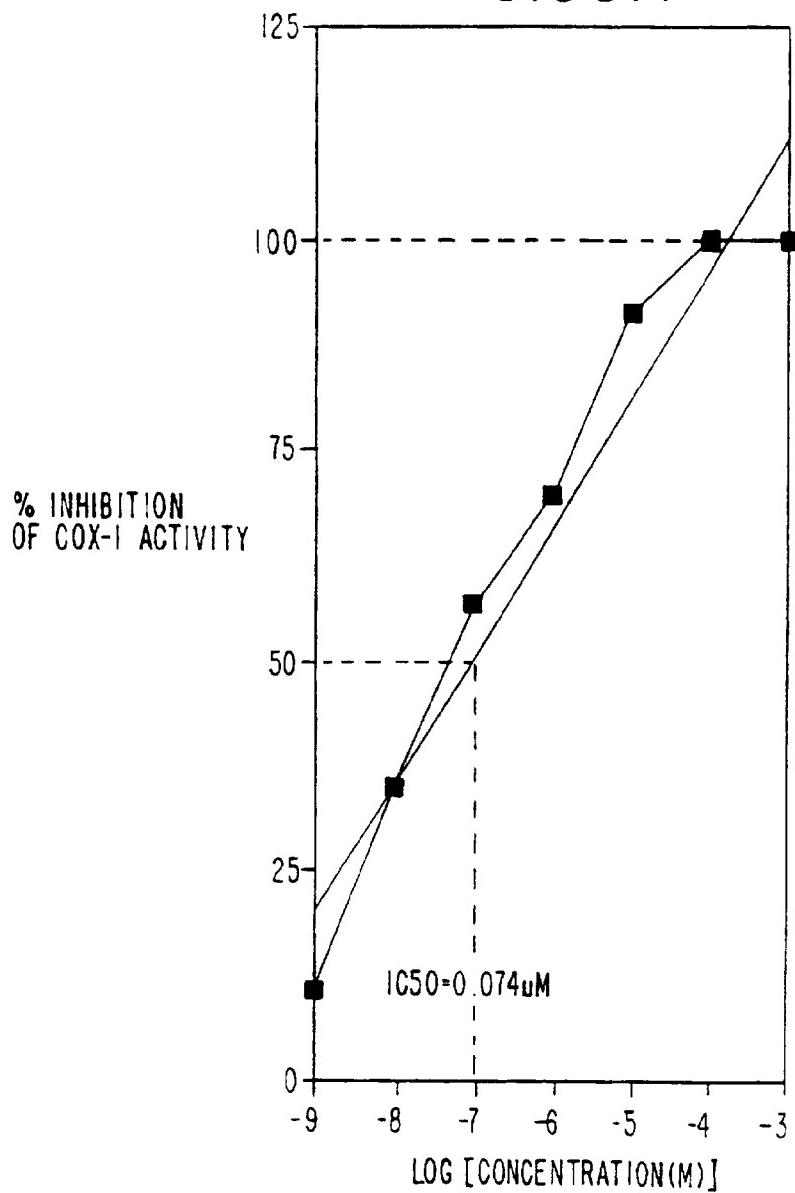
FIG. 35



(*) WITH THE EXCEPTION OF SAMPLE S11

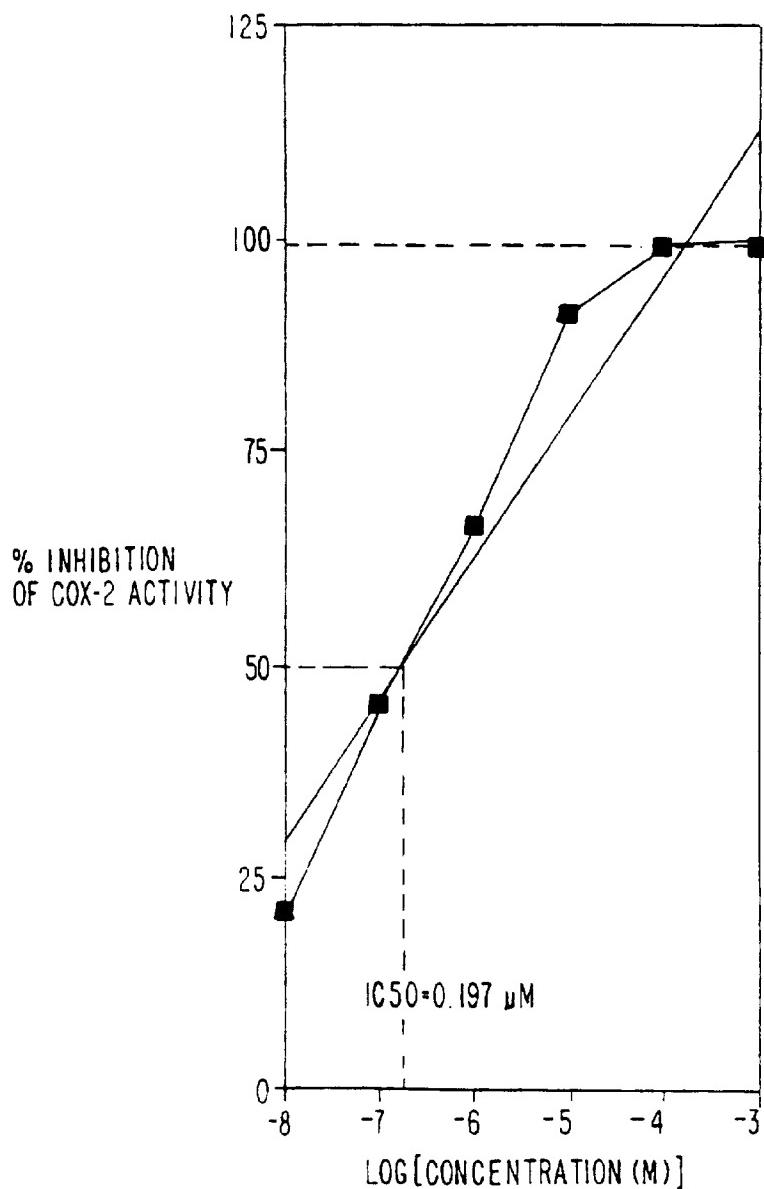
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FIG. 36A



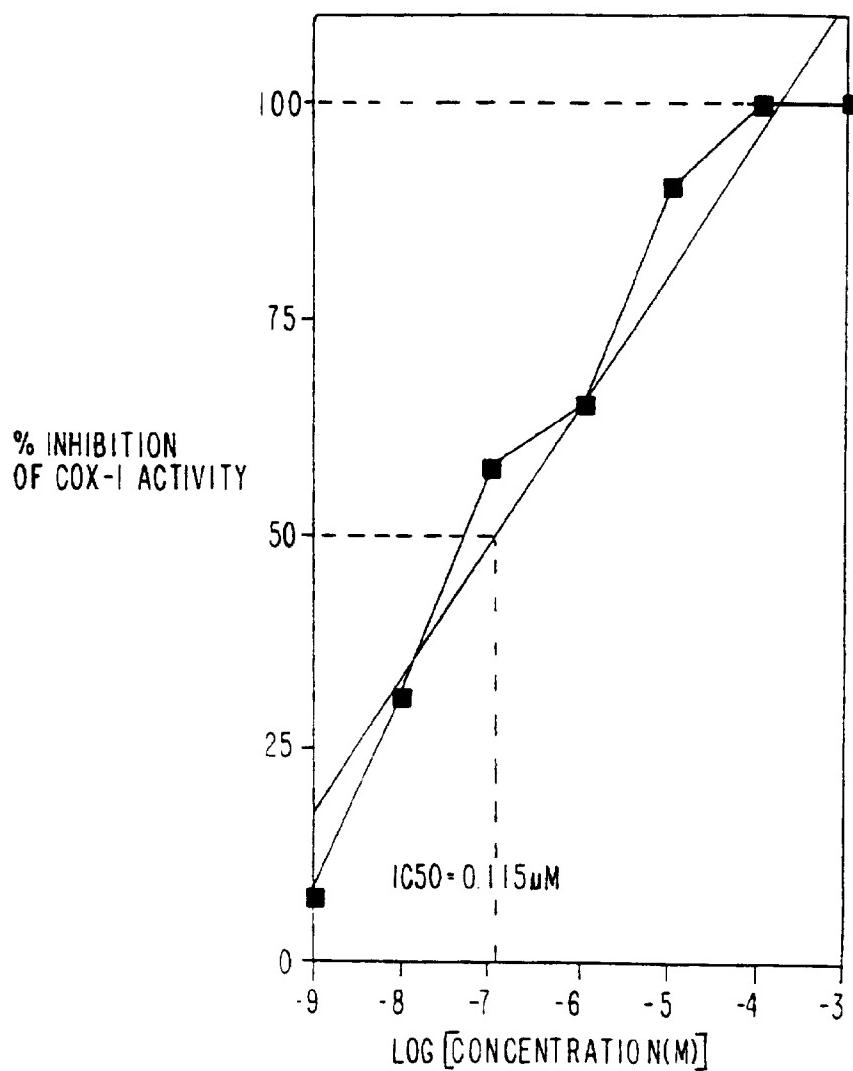
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FIG.36B



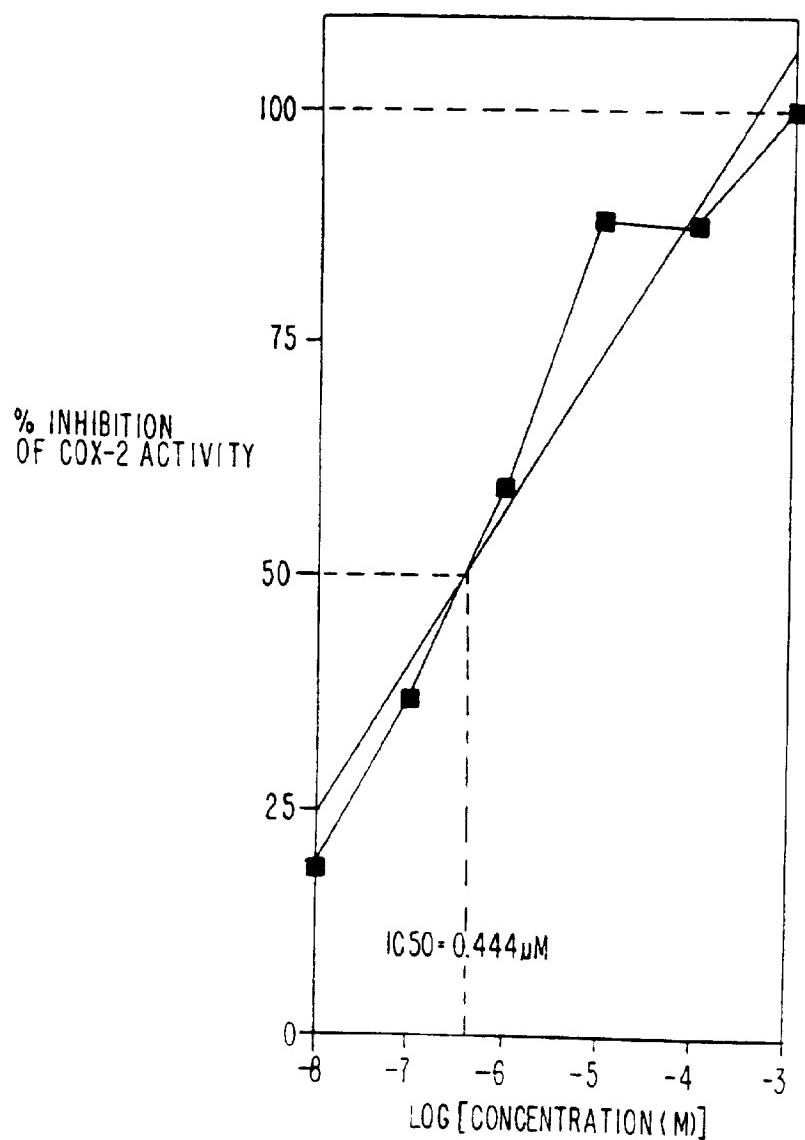
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FIG. 36C



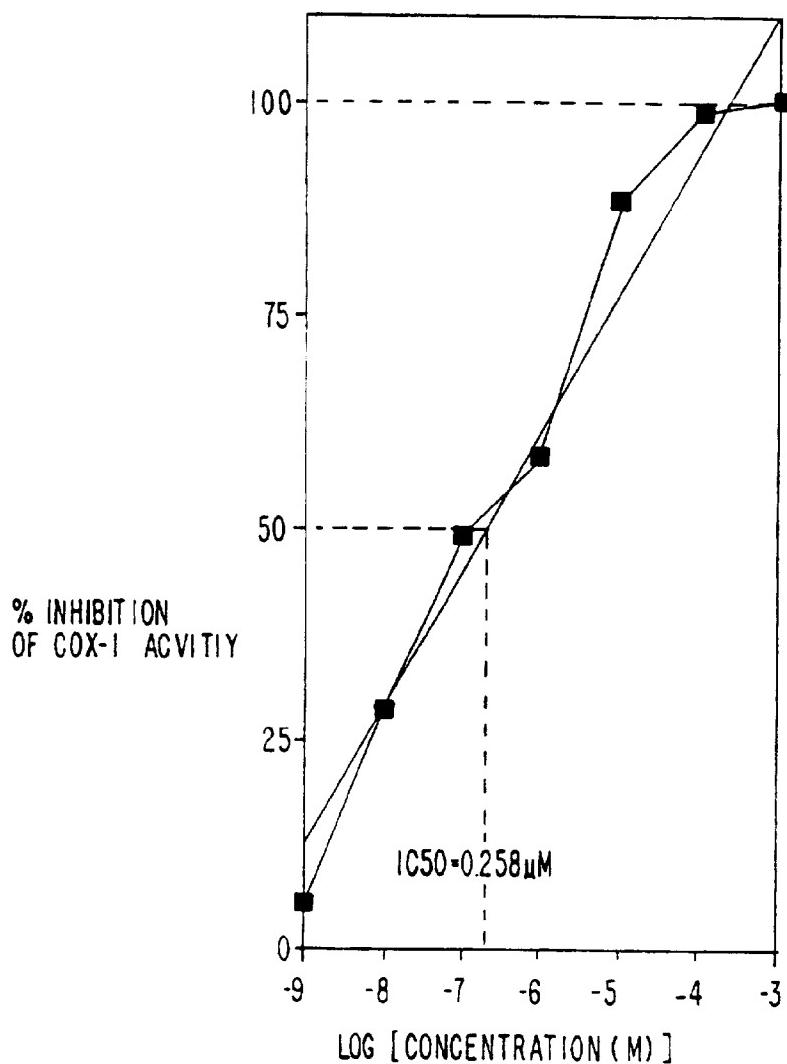
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FIG. 36D



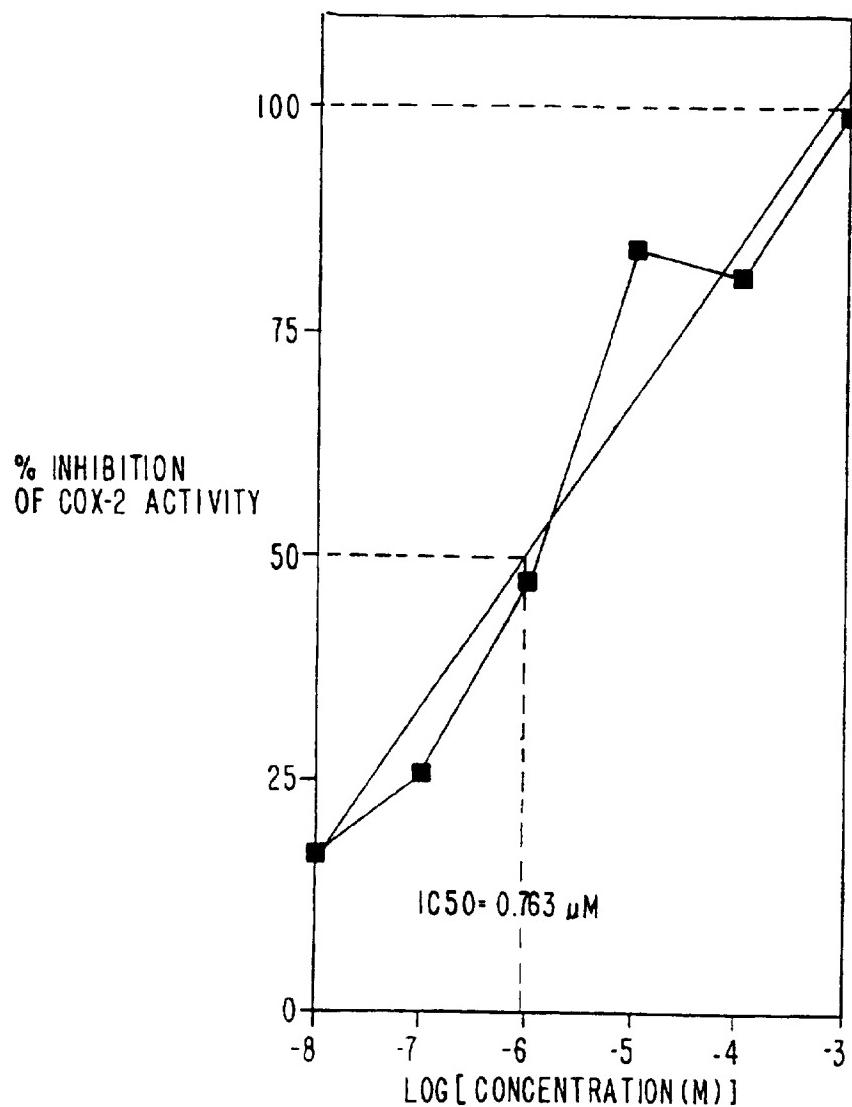
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FIG. 36E



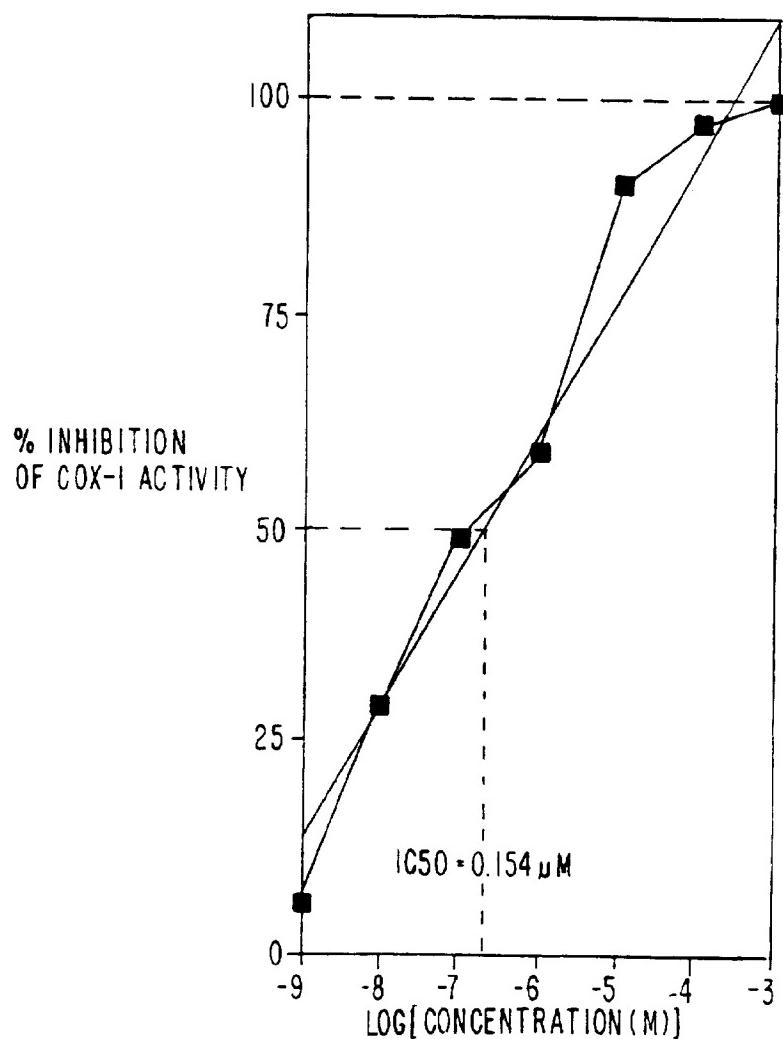
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FIG. 36F



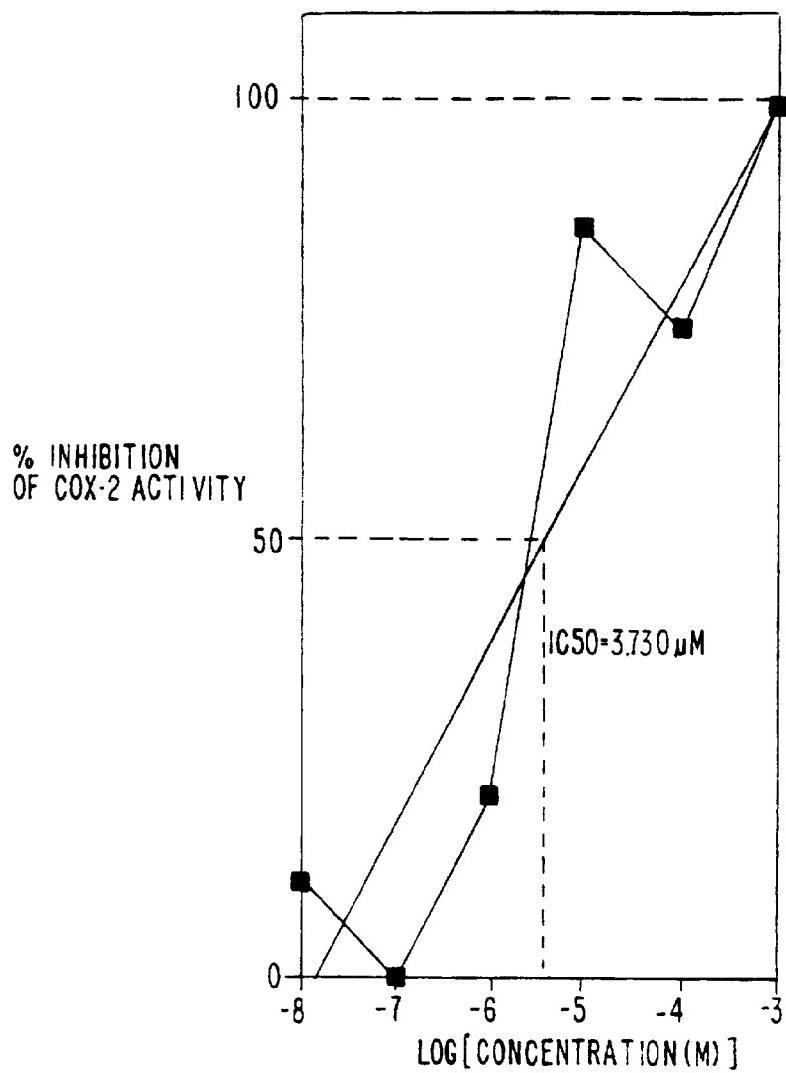
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FIG. 36G



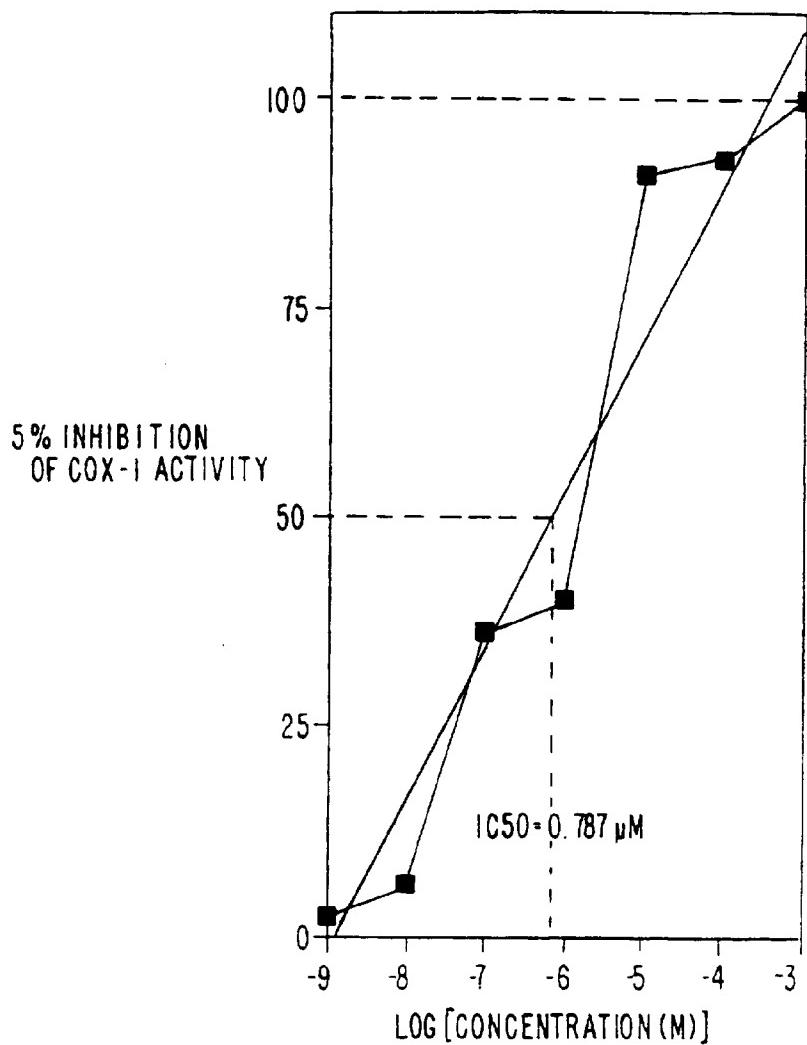
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FIG. 36H



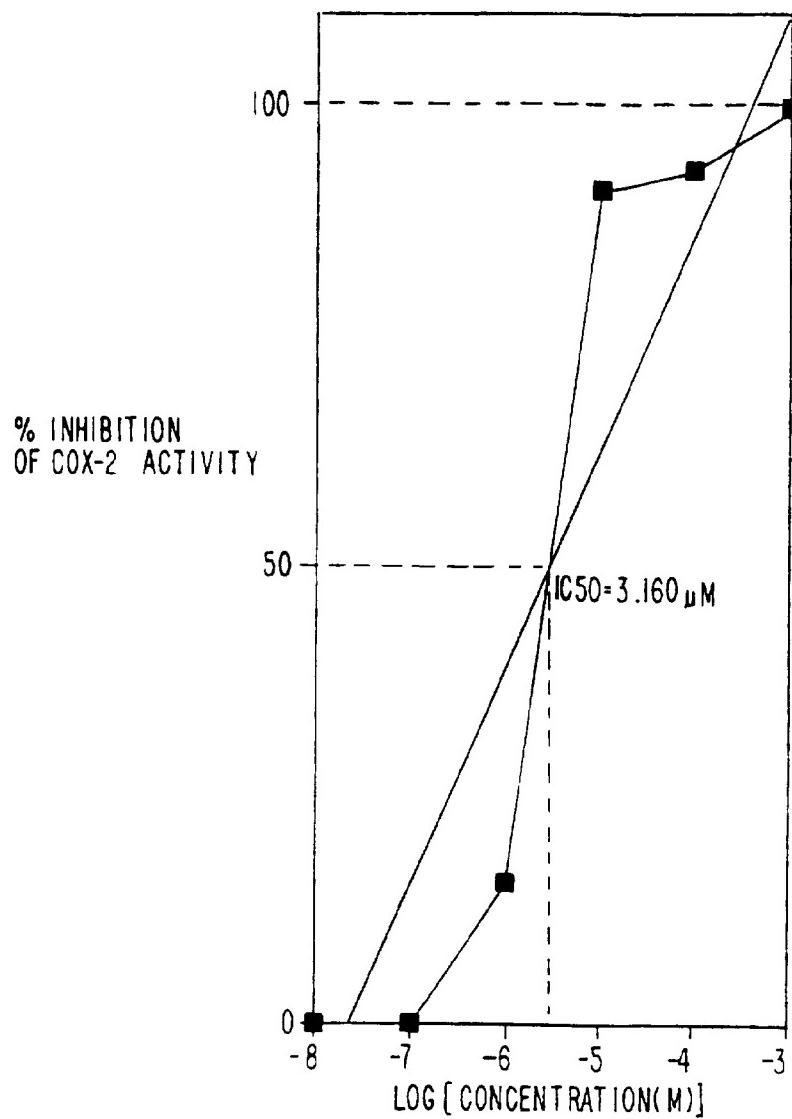
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FIG. 36I



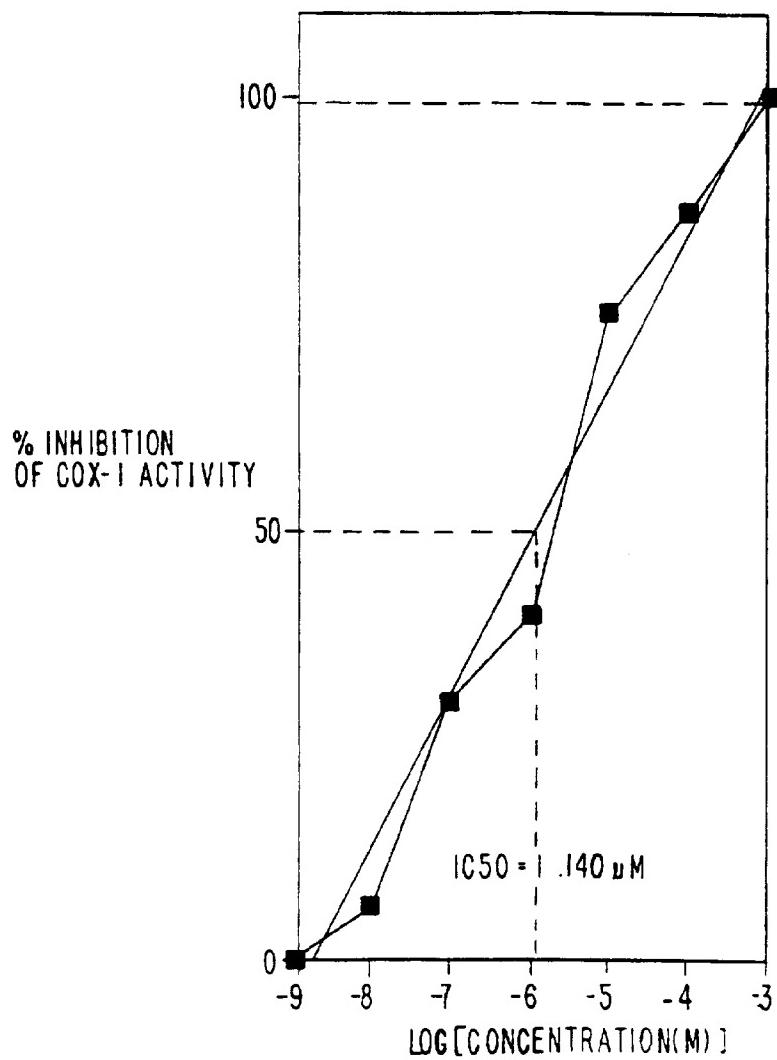
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FIG. 36J



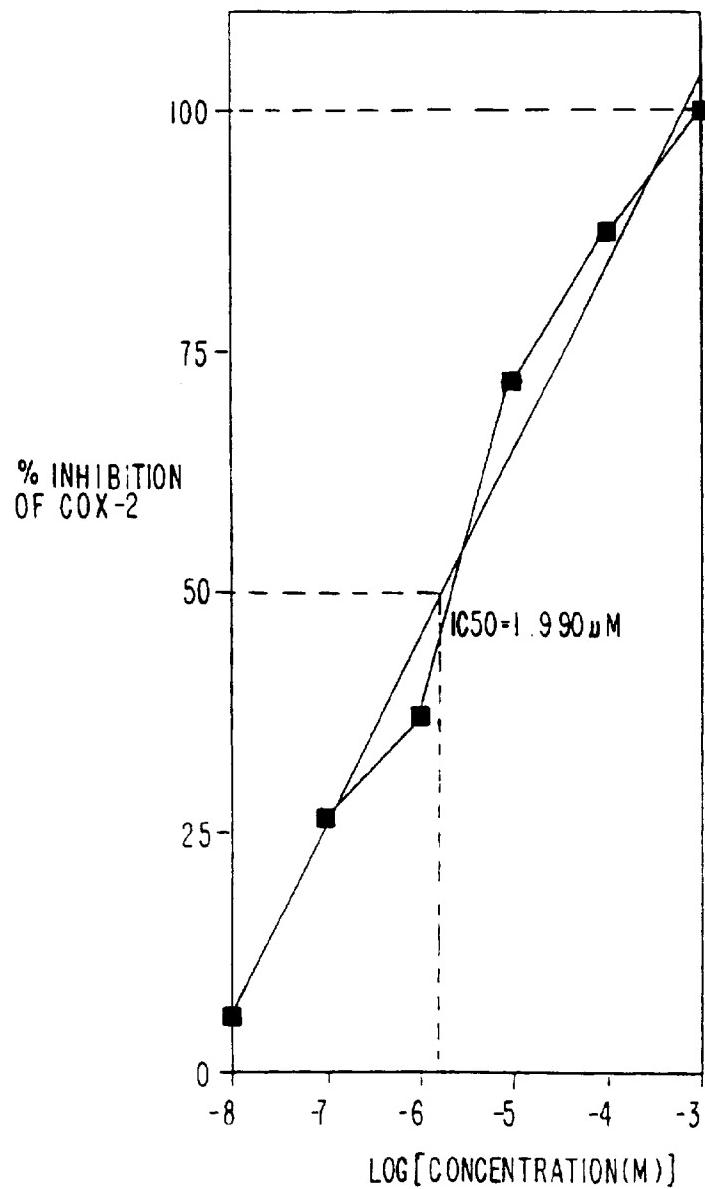
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FIG. 36K



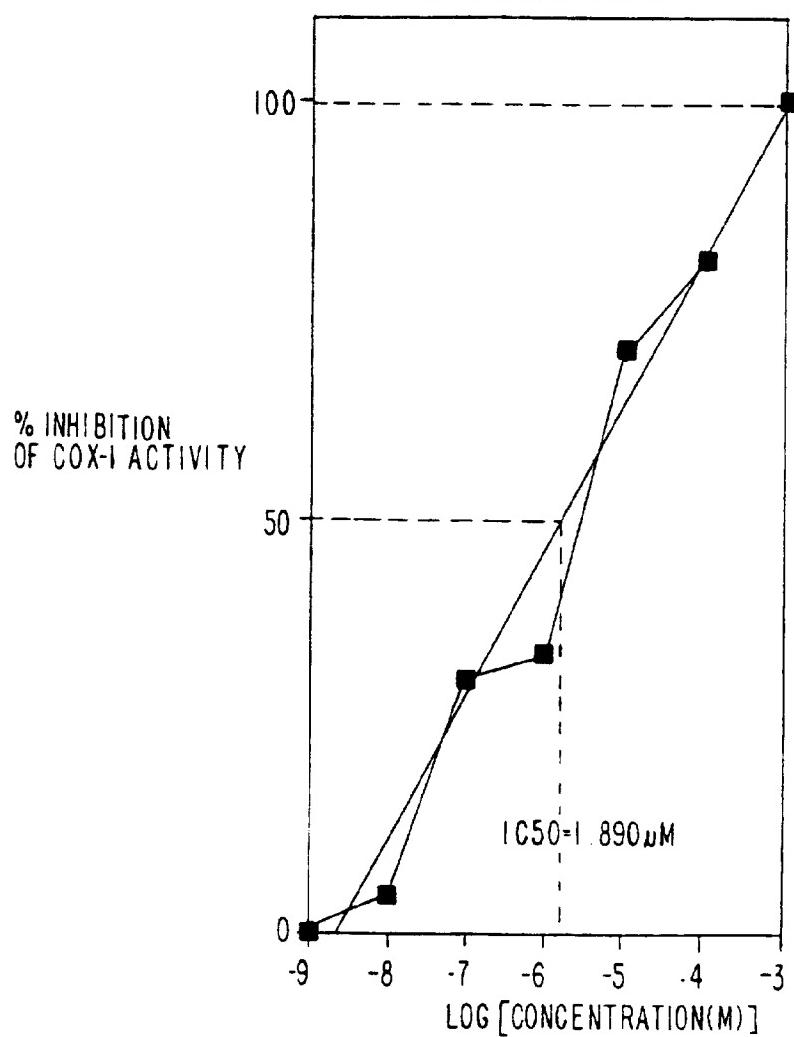
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FIG. 36L



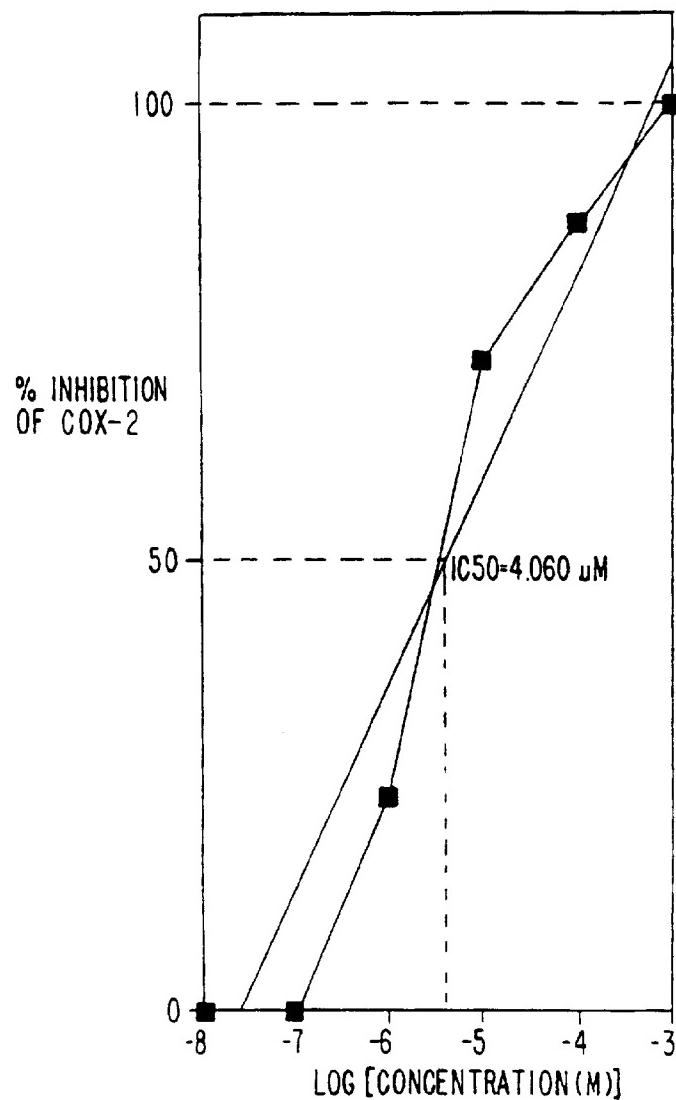
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FIG. 36M



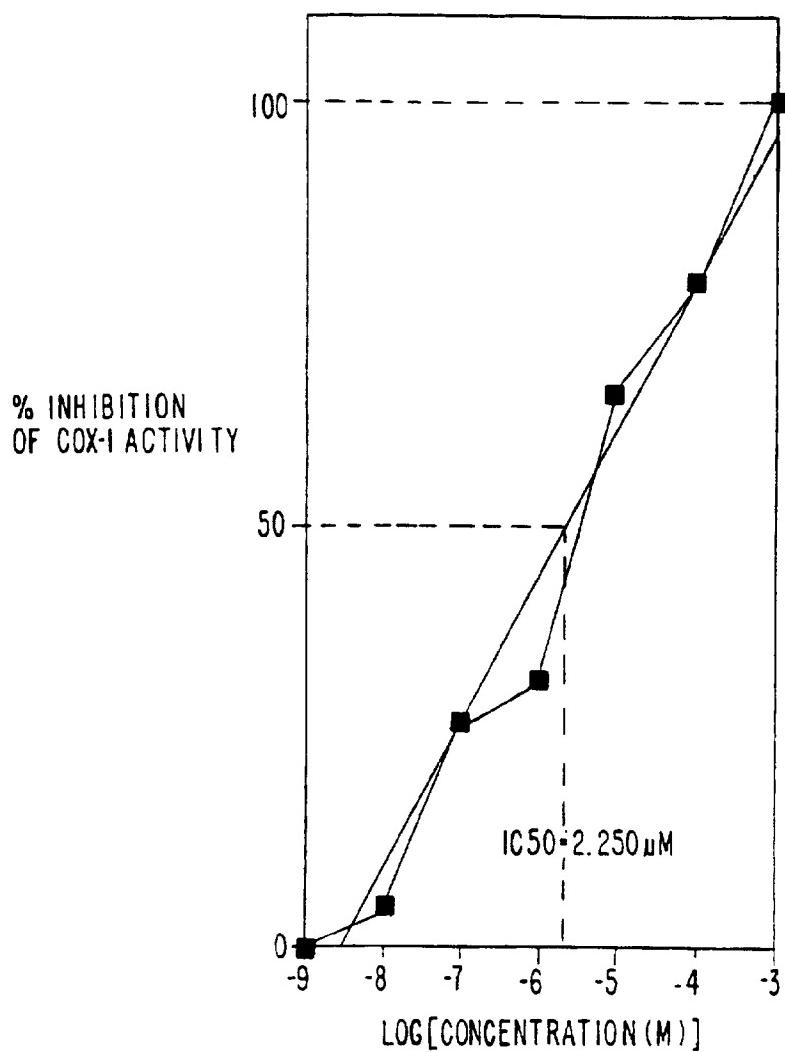
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FIG. 36N



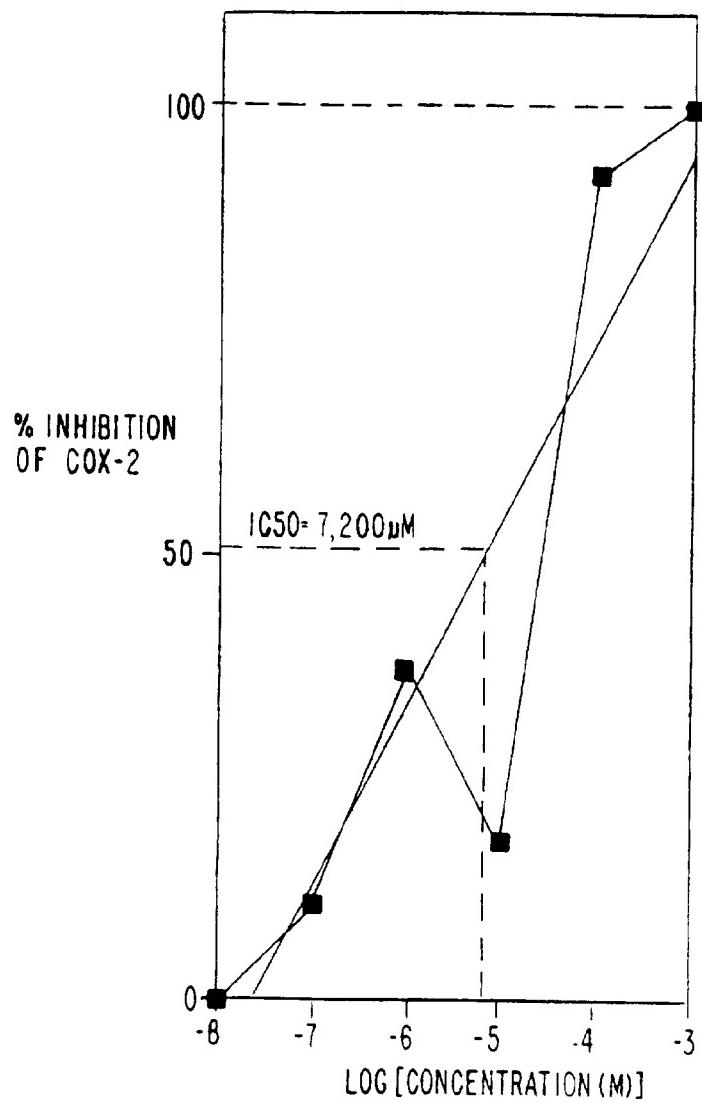
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FIG.360



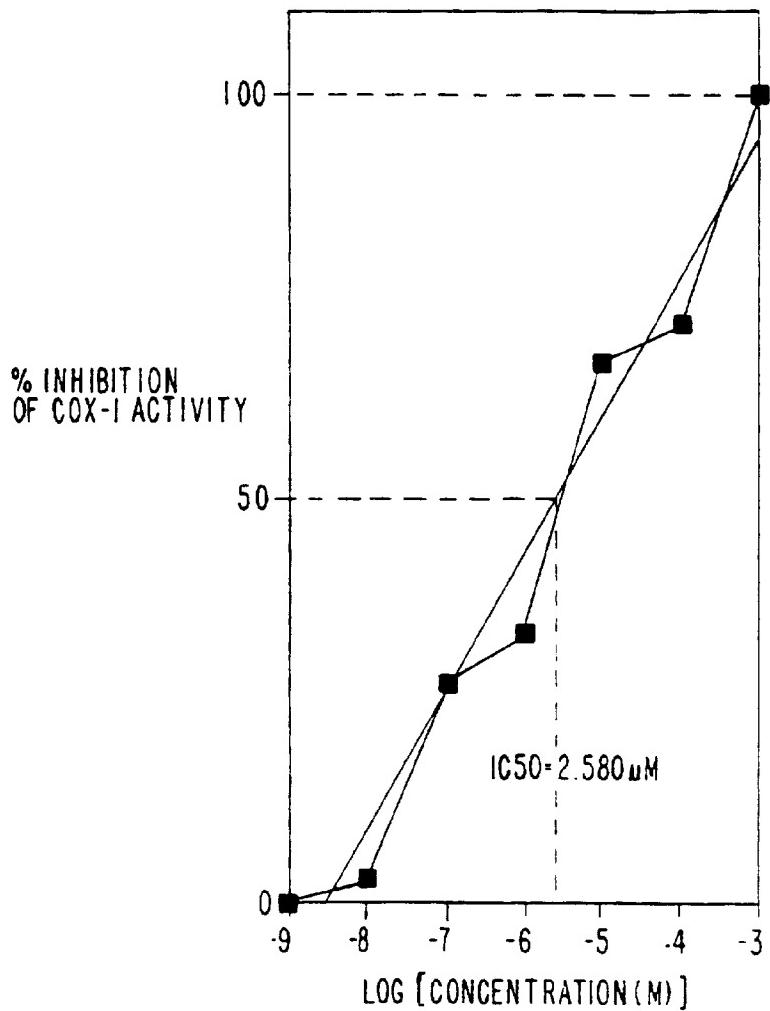
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FIG. 36P



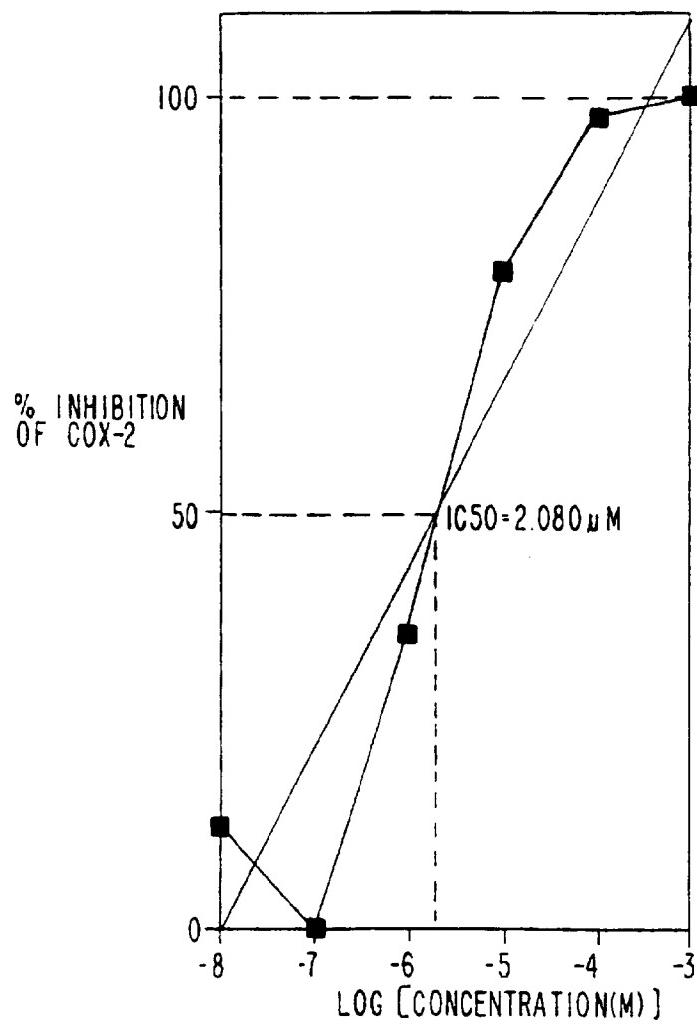
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FIG. 36Q



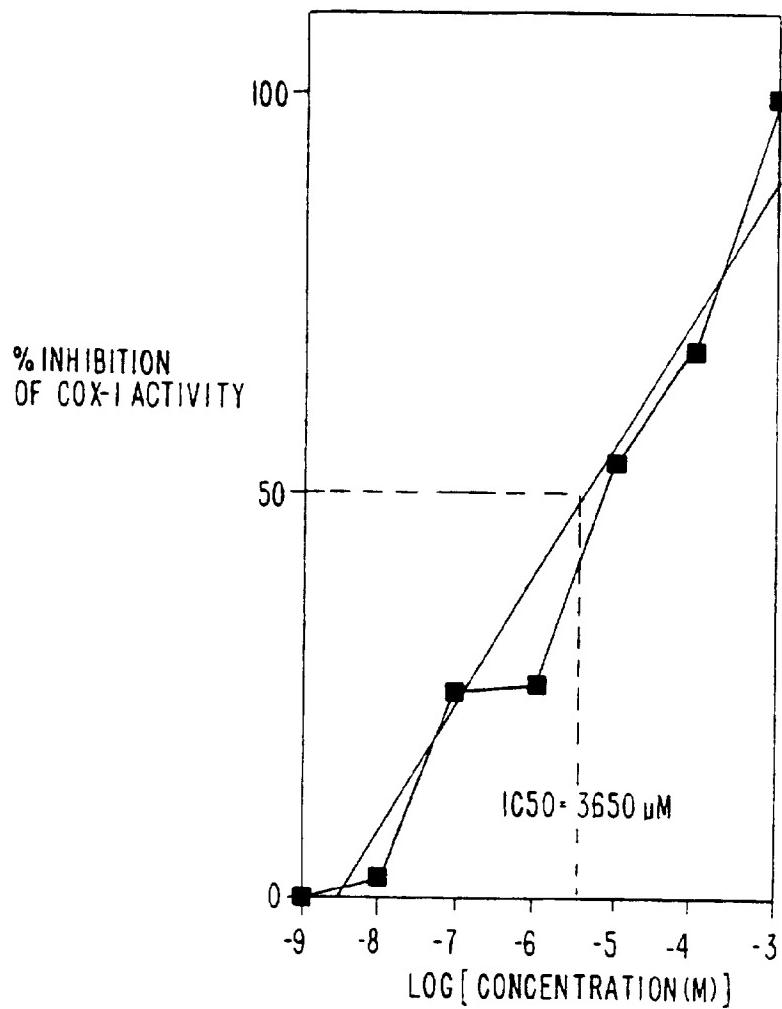
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FIG. 36R



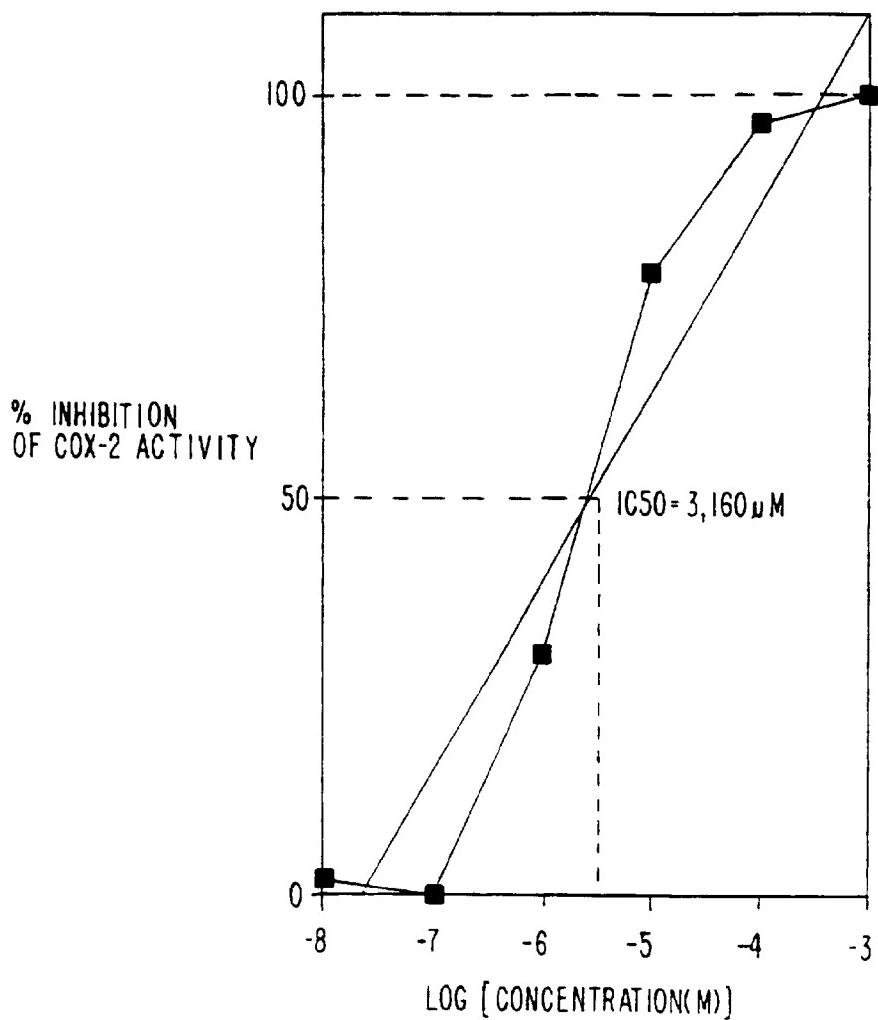
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FIG. 36S



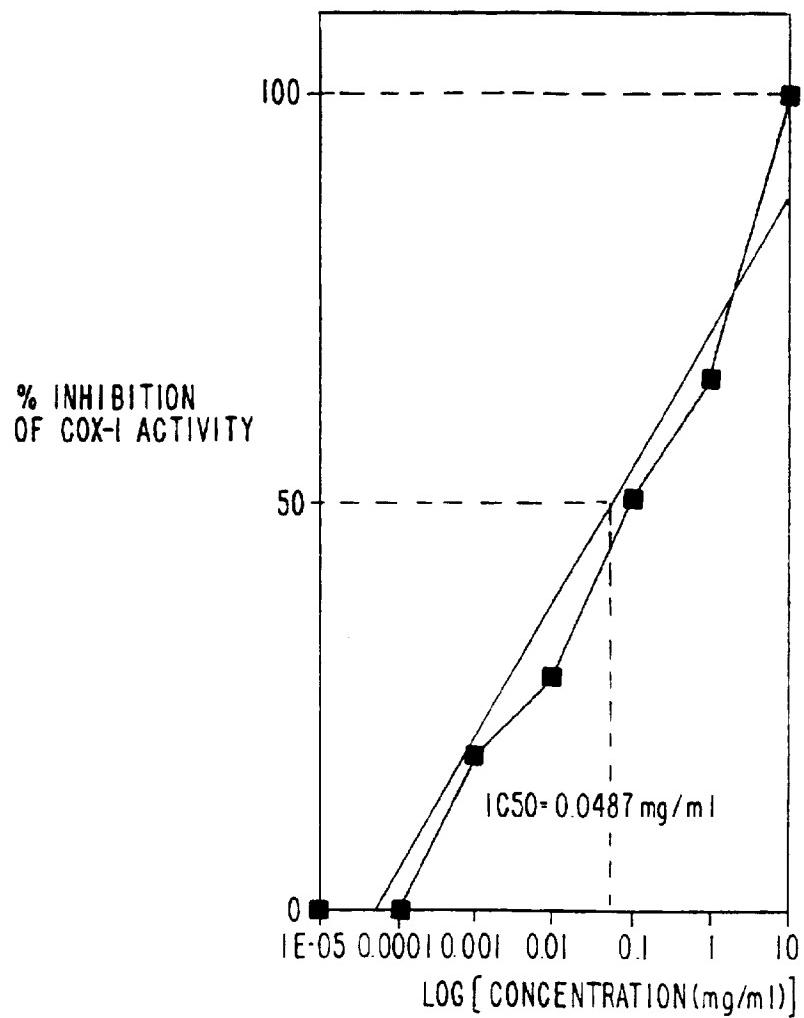
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FIG. 36T



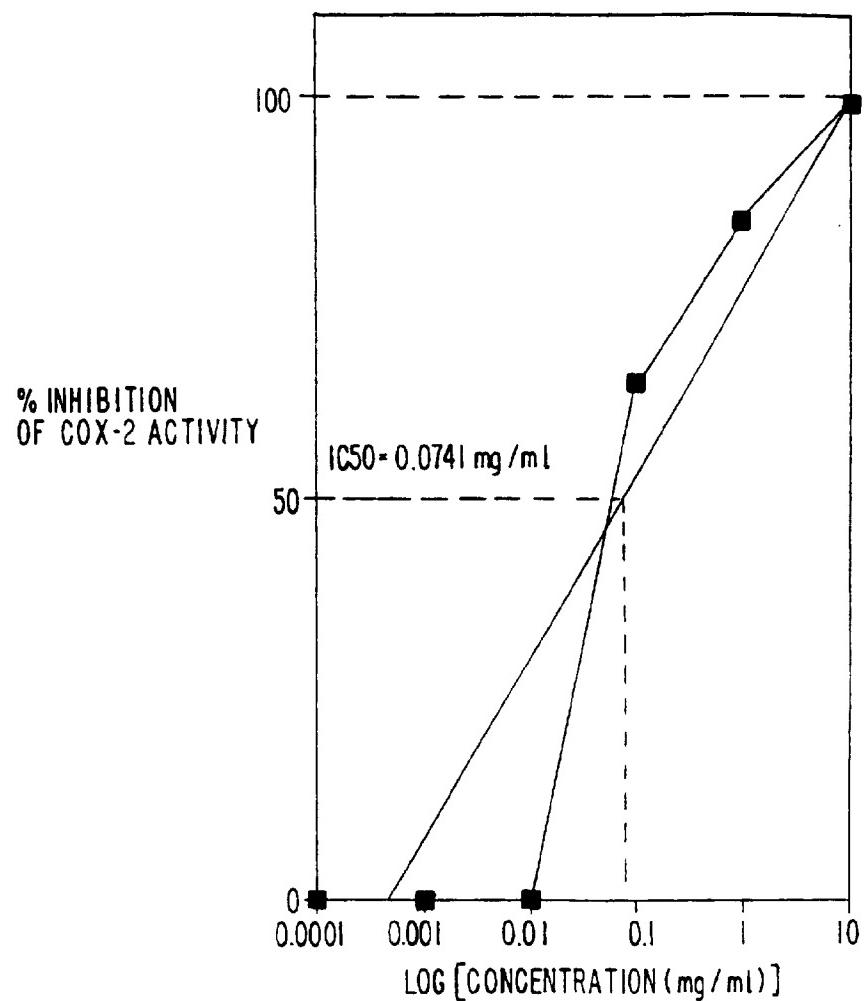
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FIG. 36U



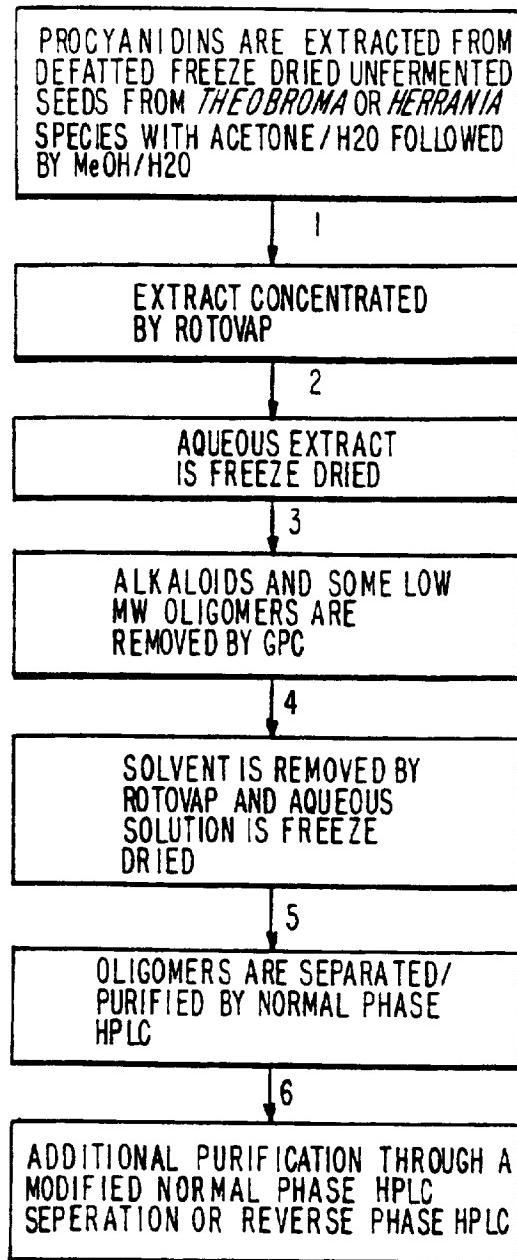
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FIG. 36V



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FIG. 37



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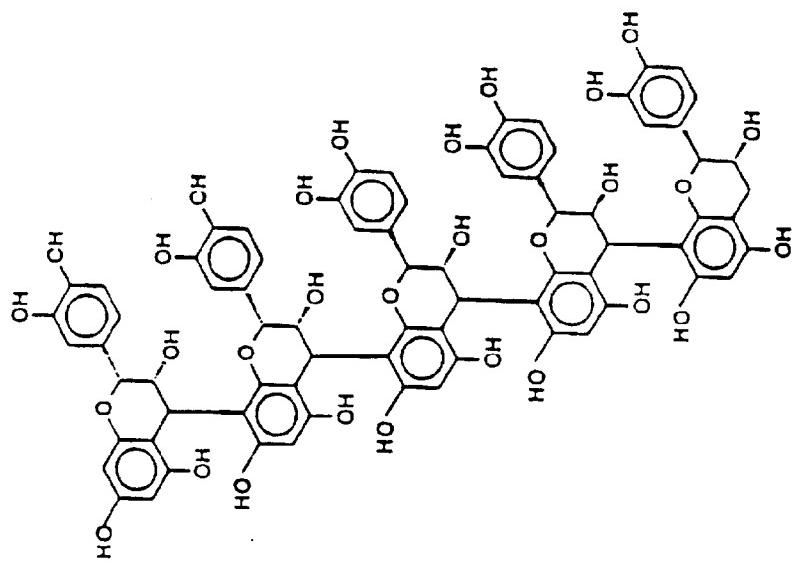


FIG. 38B

(4-8)(4-8)(4-6)CENTAMER

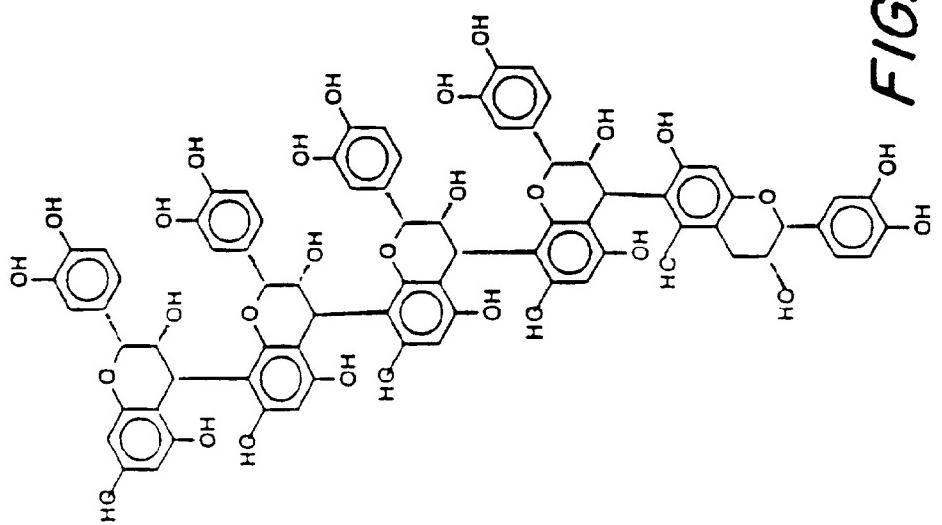
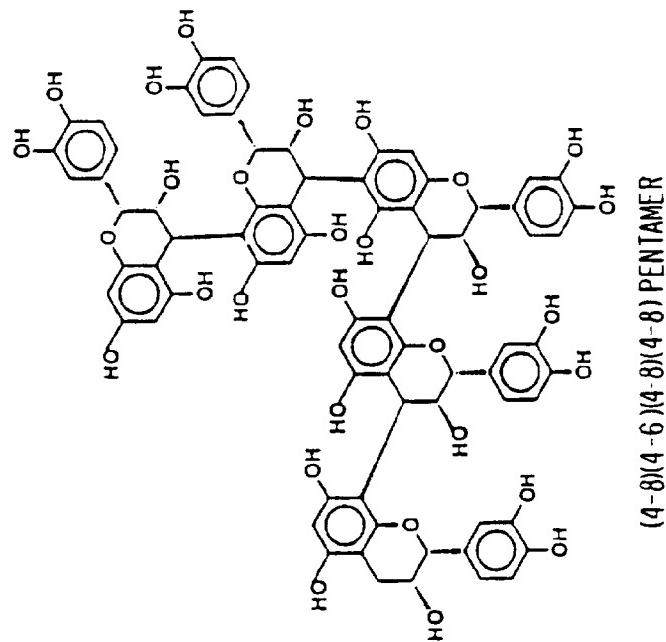


FIG. 38A

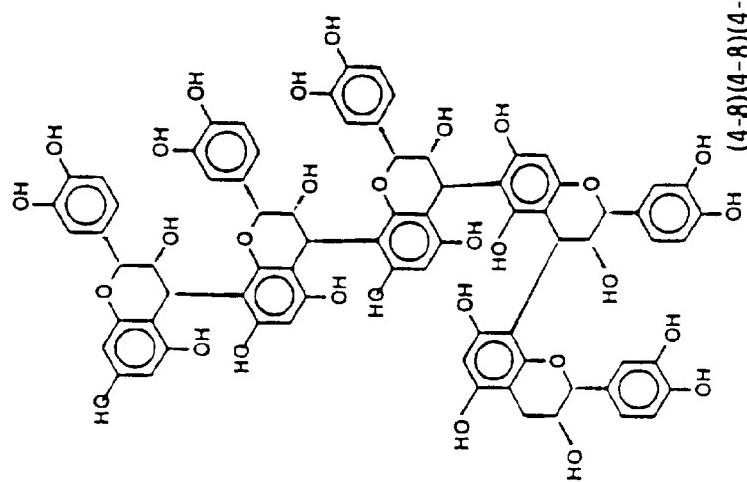
(4-8)(4-8)(4-6)PENTAMER

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(4-8)(4-6)(4-8)(4-6)PENTAMER

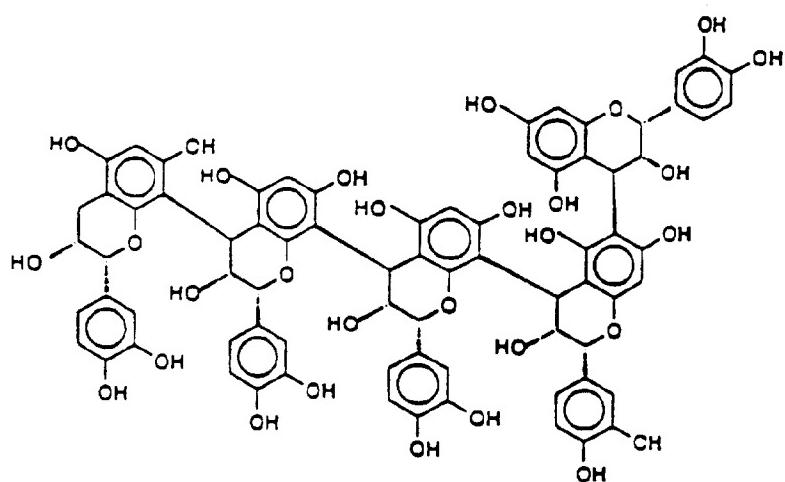
FIG. 38D



(4-8)(4-6)(4-8)(4-6)(4-6)PENTAMER

FIG. 38C

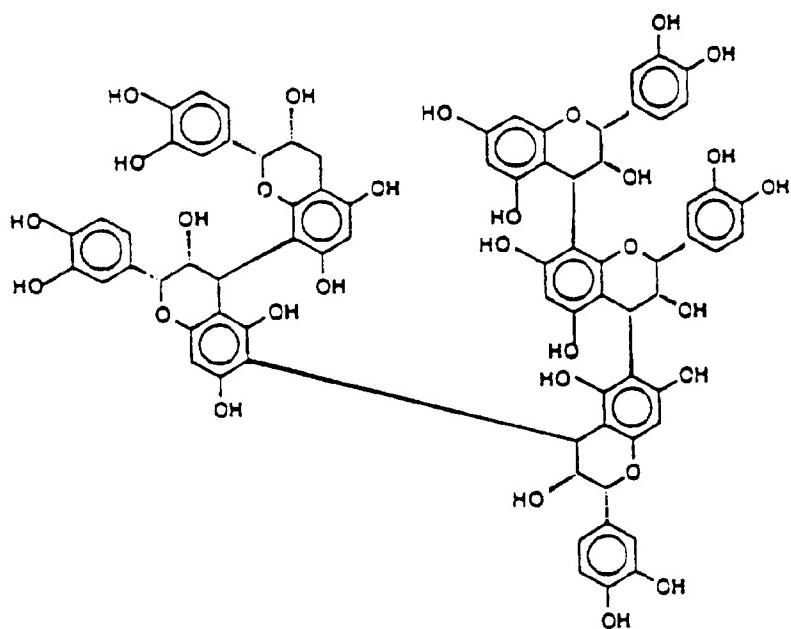
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(4-6)(4-8)(4-8)(4-8) PENTAMER

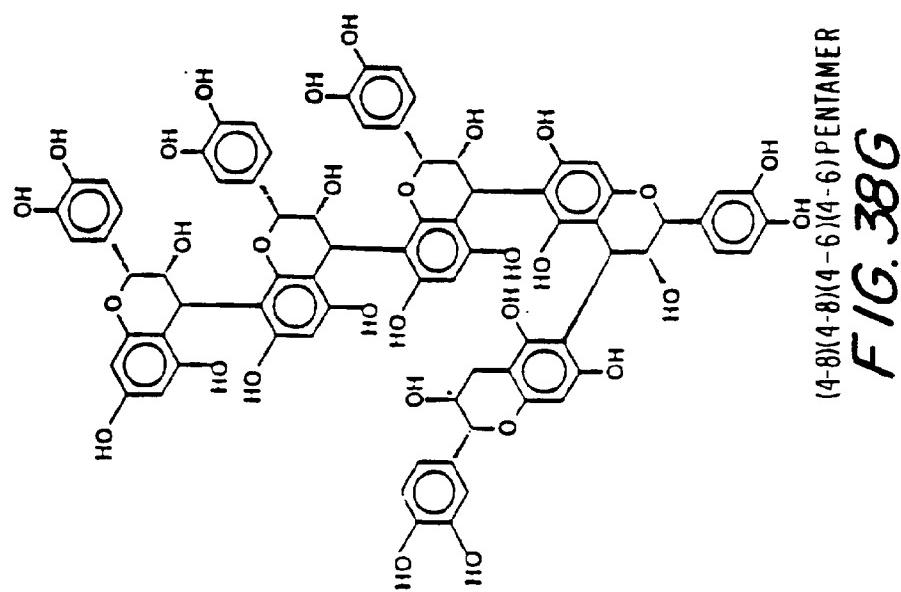
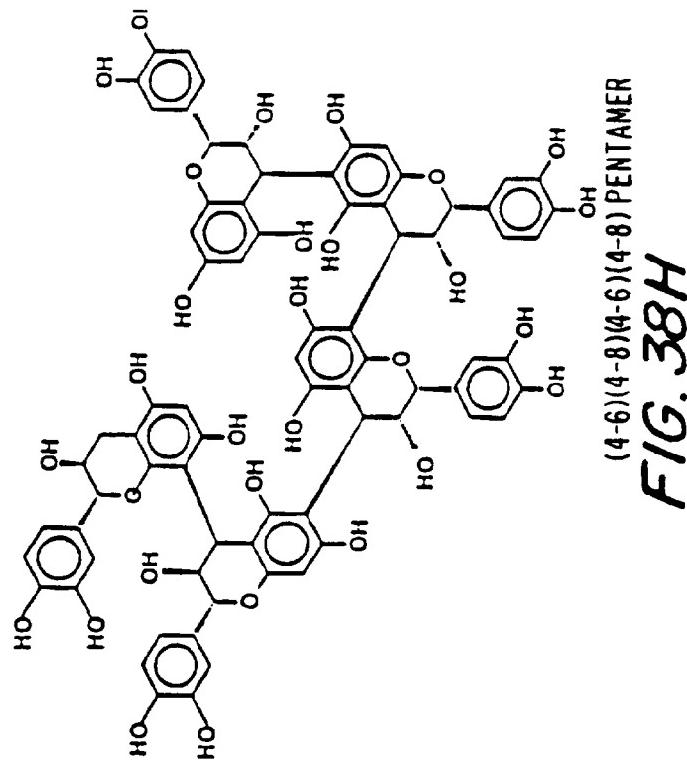
FIG. 38E

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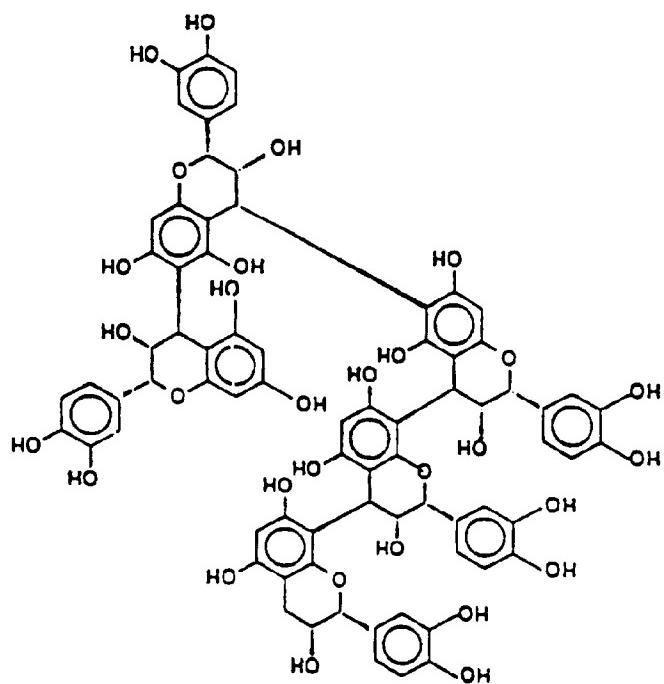


(4-8)(4-6)(4-6)(4-8) PENTAMER

FIG. 38F



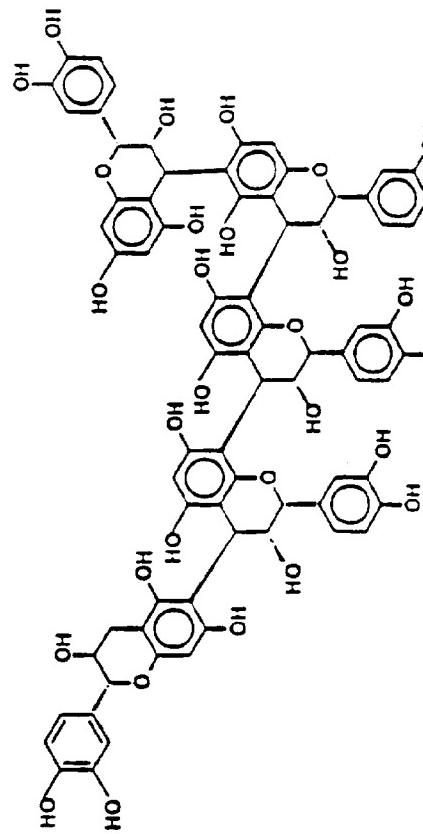
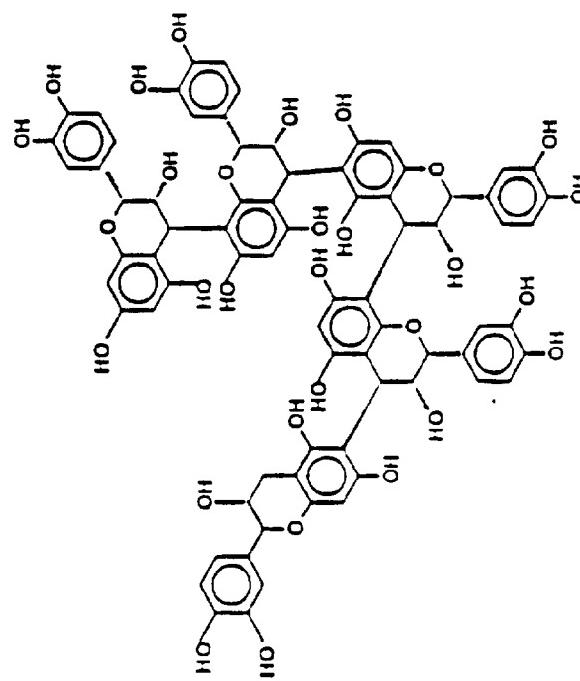
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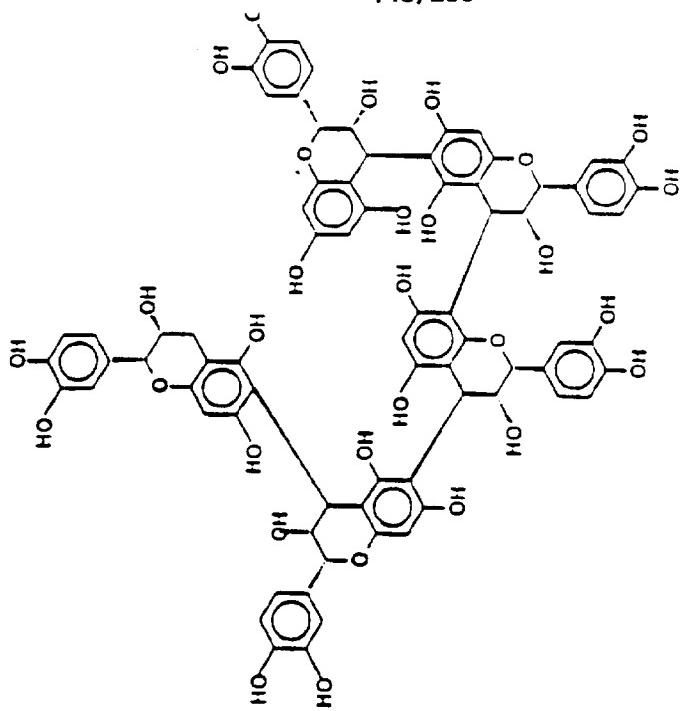
(4-6)(4-6)(4-8)(4-8)PENTAMER

FIG. 38I

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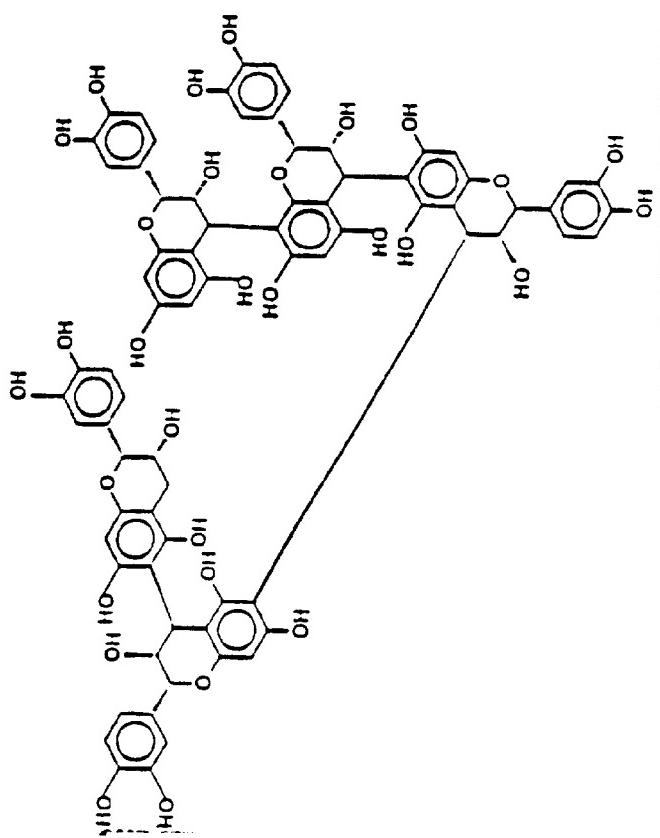
*F1G. 38K**F1G. 38J*

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(4-6)(4-8)(4-6)(4-6)PENTAMER

FIG. 38M



(4-8)(4-6)(4-6)(4-6)PENTAMER

FIG. 38L

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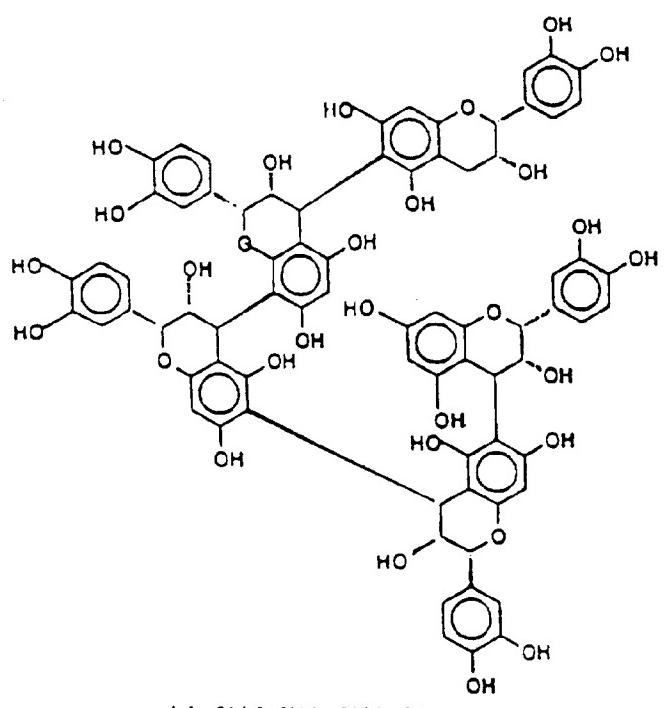
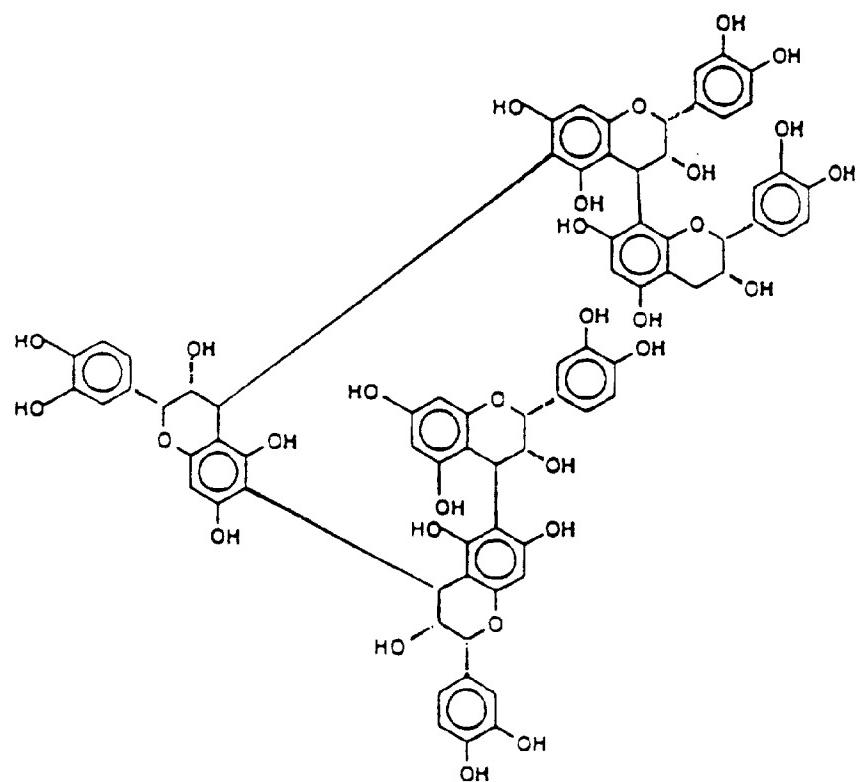


FIG. 38N

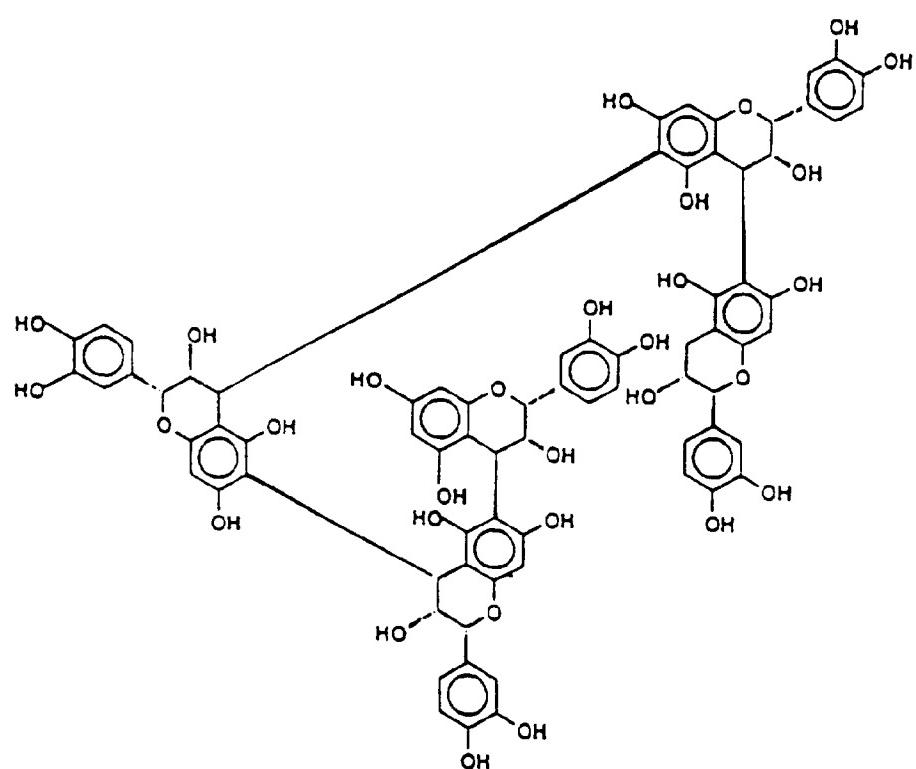
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(4-6)(4-6)(4-6)(4-6) PENTAMER

FIG. 380

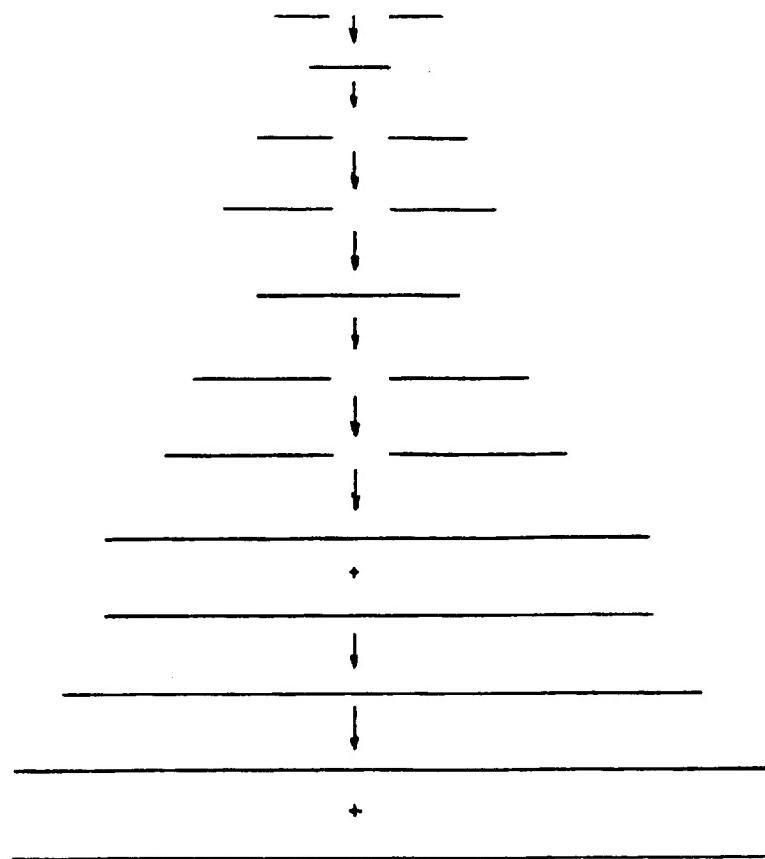
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(4-6)(4-6)(4-6)(4-8)PENTAMER

FIG. 38P

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FIG. 39A

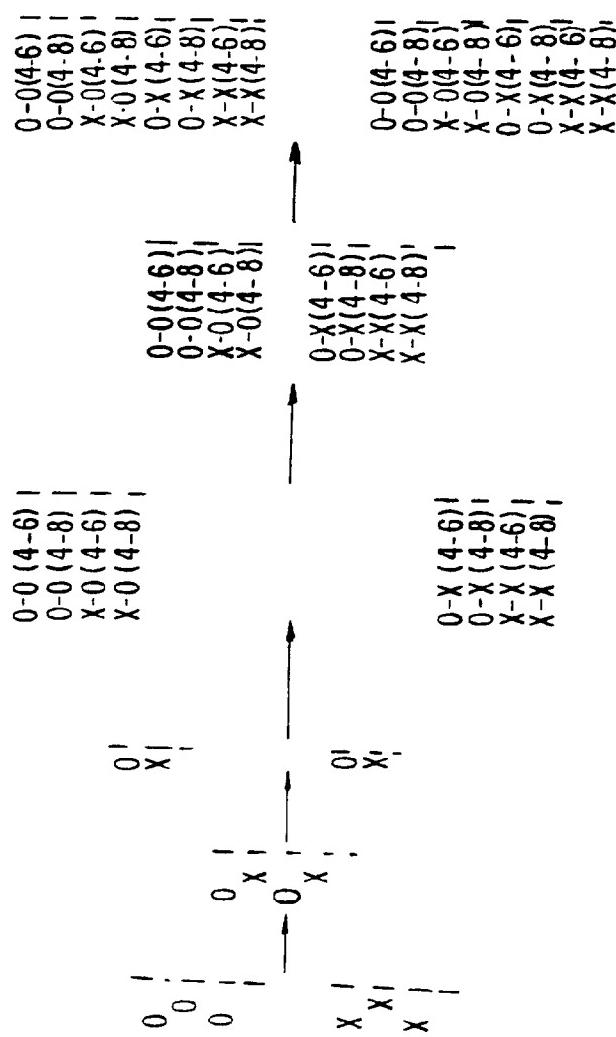
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X-CAT
0-EC

(CONTINUE LEVEL V)

FIG. 39AA

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X-CAT
0-ECF/G. 39B
LEVEL I

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X = CAT
O = ECO

(LEVEL 1)

FIG. 39 C

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X-CAT
0-EC

FIG. 39D

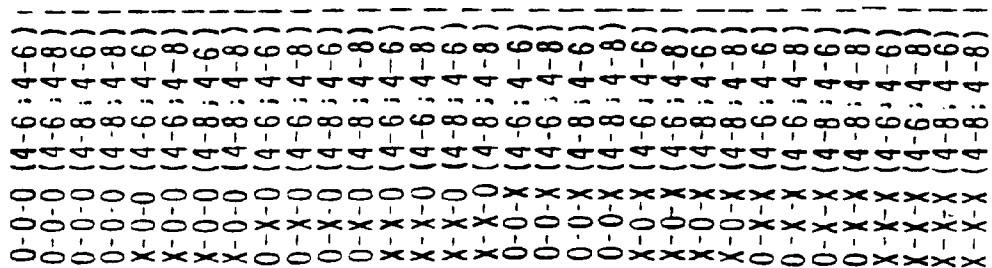
X-X-X (4-8; 4-6);
X-X-X (4-8; 4-8);

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X = CAT
0 = EC

(LEVEL II)

FIG. 39E



X=CAT
O=EC

(CONTINUE-LEVEL II)

FIG. 39F

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X = CAT
0 = EC

三

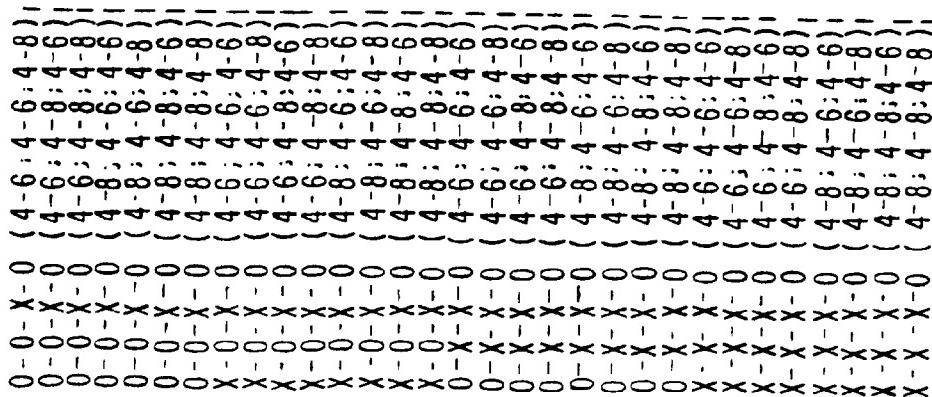
FIG. 396

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X=CAT
0=EC

(CONTINUE LEVEL III)

FIG. 39H



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X=CAT
O=EC

(CONTINUE ON LEVEL III)

X-X-0-X (4-8; 4-8; 4-8);

A grid of musical notes and rests on a staff. The notes are represented by vertical stems with small circles at the top, and rests are represented by vertical dashes. The grid consists of 12 columns and 10 rows. The first two columns contain mostly rests. Columns 3 through 6 contain mostly notes. Columns 7 through 10 contain mostly rests. The last two columns contain mostly notes. The grid is bounded by horizontal dashed lines at the top and bottom.

FIG. 391

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$$X = CAT$$
$$0 = E C$$

(CONTINUE LEVEL (III))

FIG. 39J

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X-CAT
0-EC

FIG. 39K

X-X-X-X (4-8; 4-6; 4-6)
X-X-X-X (4-8; 4-6; 4-8)
X-X-X-X (4-8; 4-8; 4-6)
X-X-X-X (4-8; 4-8; 4-8)

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$$X = CAT$$

$$O = ECG$$

(LEVEL IV)

FIG. 39L

X = CAT
0 = EC

CONTINUE-LEVEL IV

FIG. 39M

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X = CAT
O = EC

(CONTINUE LEVEL IV)

FIG. 39N

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$$X = CAT$$
$$0 = EC$$

(CONTINUE LEVEL IV)

F/G. 39 0

168/235

X = CAT
0 = EC

(CONTINUE LEVEL IV)

FIG. 39P

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$$\begin{array}{l} X = CAT \\ 0 = EC \end{array}$$

(CONTINUE LEVEL IV)

FIG. 390

170/235

$$X = CAT$$

$$0 = EC$$

(CONTINUE LEVEL IV)

FIG. 39R

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X-CAT
O-EC

(CONTINUE LEVEL IV)

FIG. 395

A large grid of handwritten digits and symbols. The grid consists of 10 columns and 10 rows. Each cell contains one of the following characters: '0', '1', '2', '3', '4', '5', '6', '7', '8', '9', or 'X'. The characters are written in a cursive, black-and-white style. The '0's are at the bottom left, followed by '1's, then '2's, '3's, '4's, '5's, '6's, '7's, '8's, and finally '9's at the top right. Interspersed among these digits are numerous 'X' characters, which are distributed across the entire grid in a somewhat random pattern.

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X = CAT
O = EC

(LEVEL V)

FIG. 39T

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X = CAT
0 = EC

(CONTINUE LEVEL V)

FIG. 39U

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X-CAT
0=EC

(CONTINUE LEVEL V)

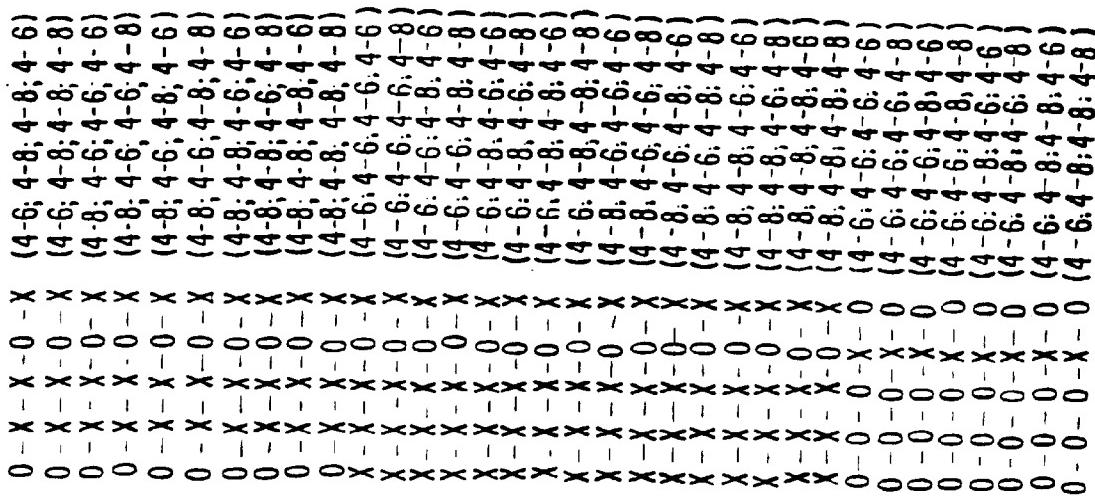
FIG. 39V

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X = CAT
0 = EC

(CONTINUE LEVEL V).

FIG. 39W



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X-CAT
O-EC

(CONTINUE LEVEL V)

FIG. 39 X

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X = CAT
0 = EC

(CONTINUE LEVEL V)

FIG. 39Y

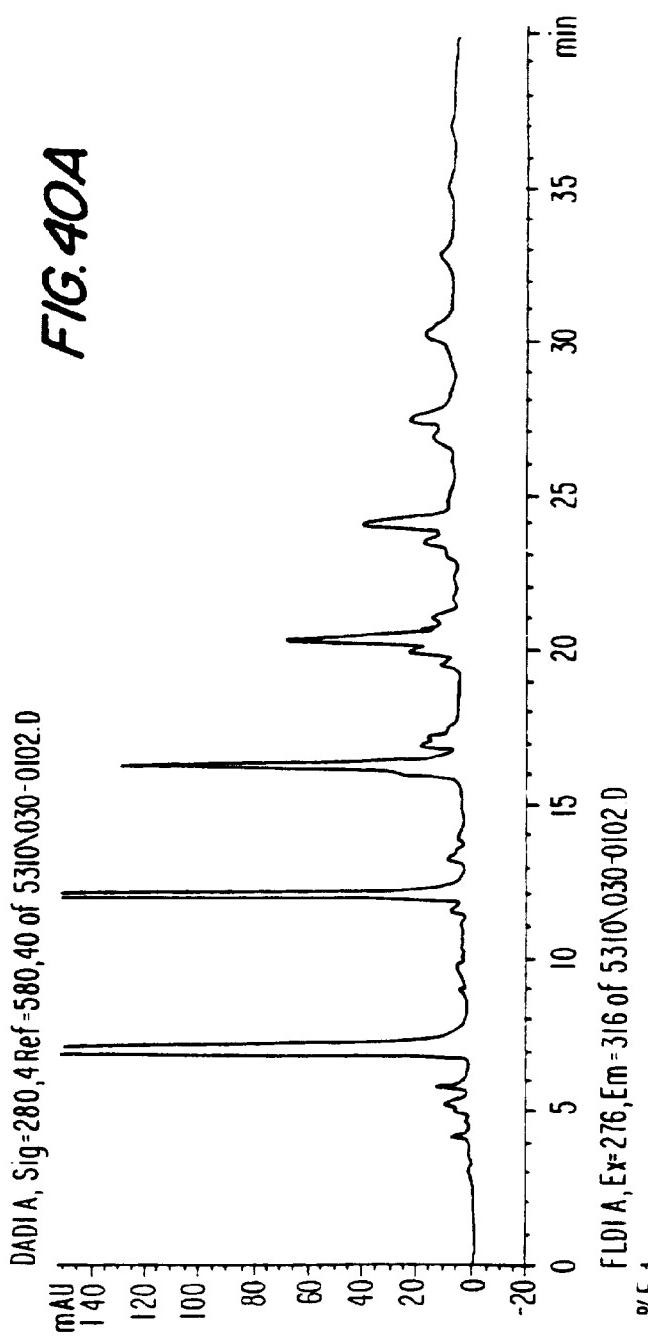
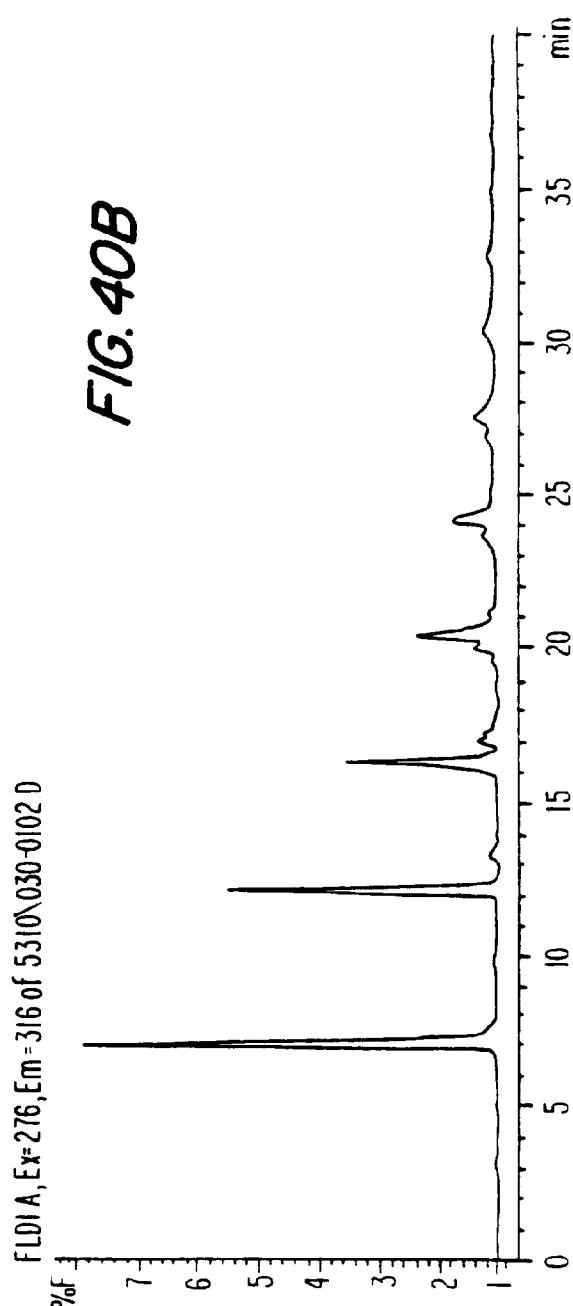
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X=CAT
O=EC

(CONTINUE LEVEL V)

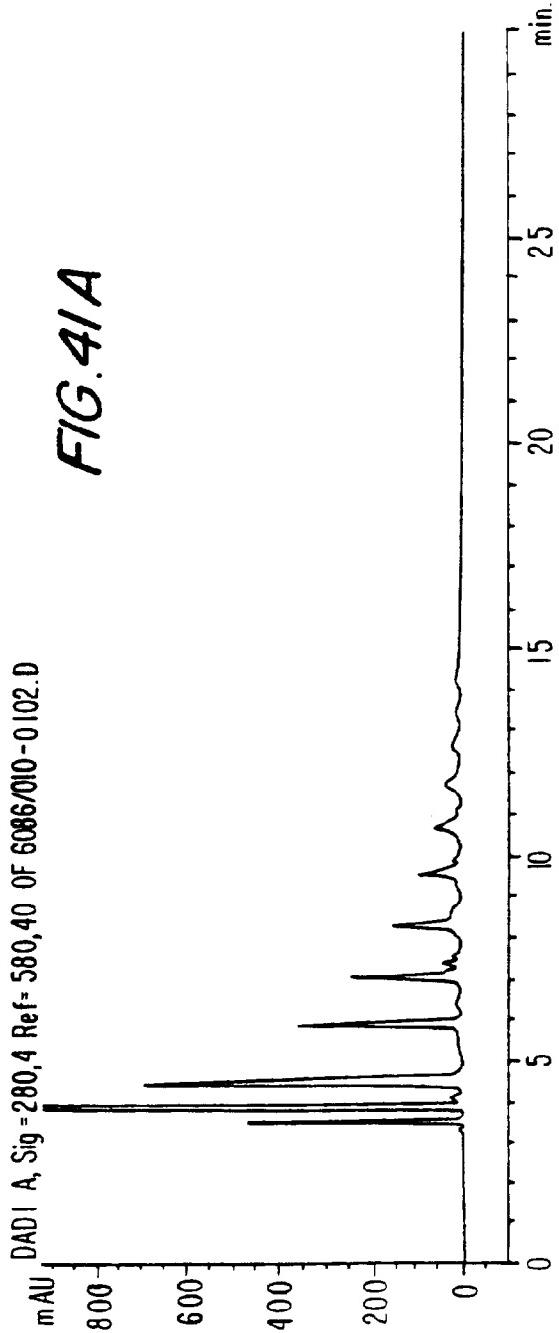
FIG. 39Z

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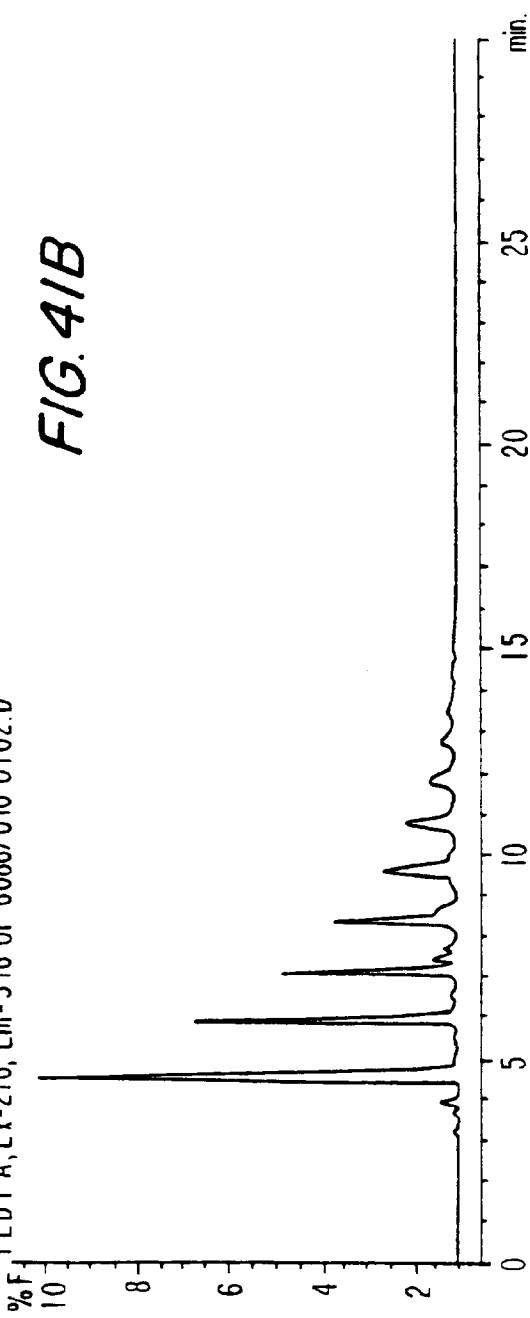
FIG. 40A*FIG. 40B*

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DAD | A, Sig = 280,4 Ref= 580,40 OF 6086/010-0102.D

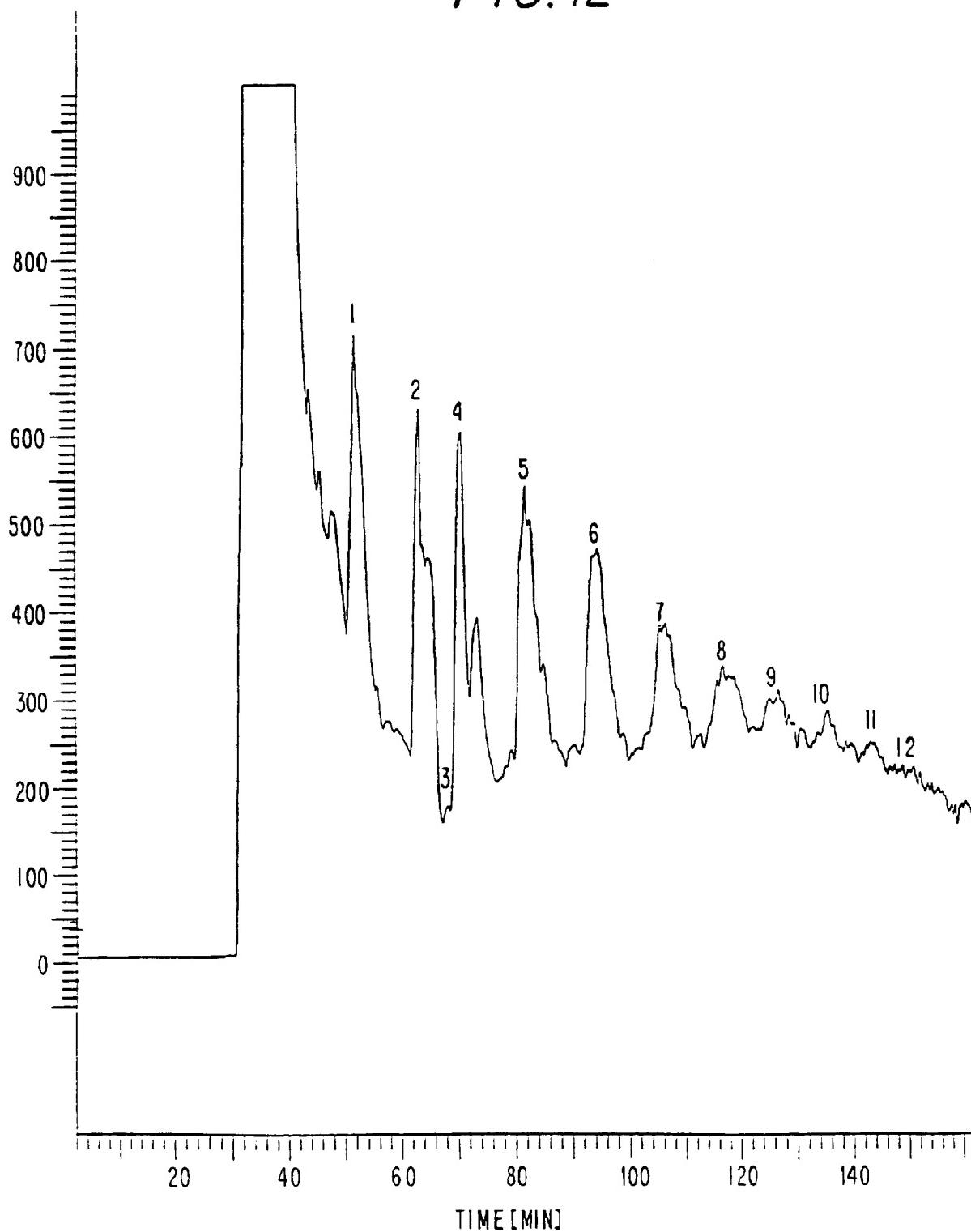
FIG. 4/A

FLDI A, Ex=276, Em=316 OF 6086/010-0102.D

FIG. 4/B

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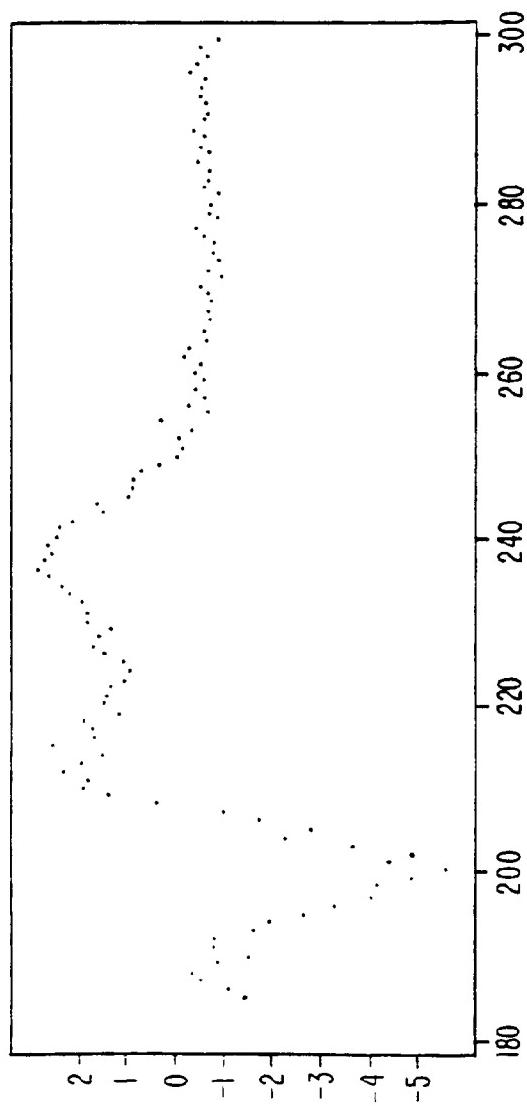
FIG.42



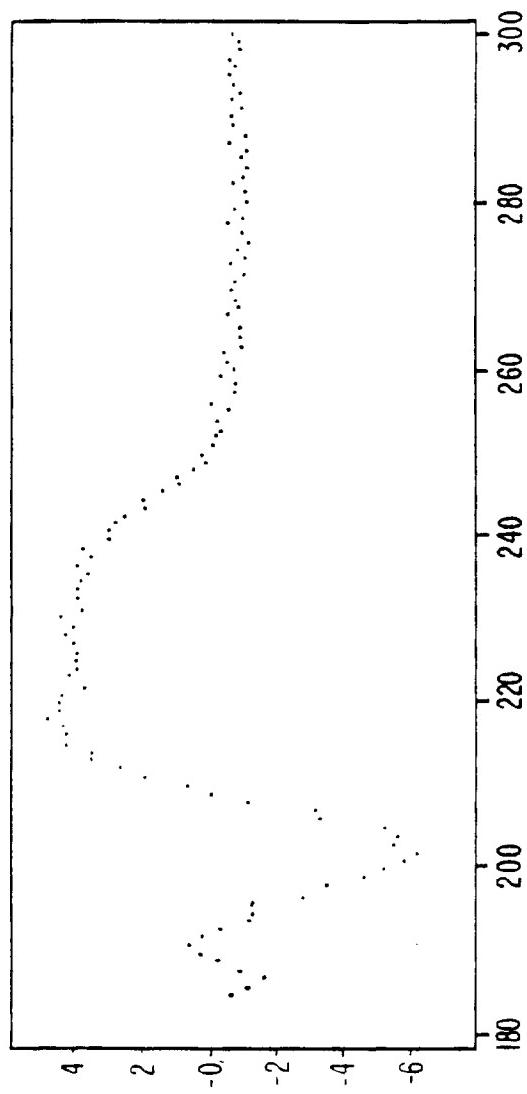
SUBSTITUTE SHEET (RULE 26)

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FIG. 43A

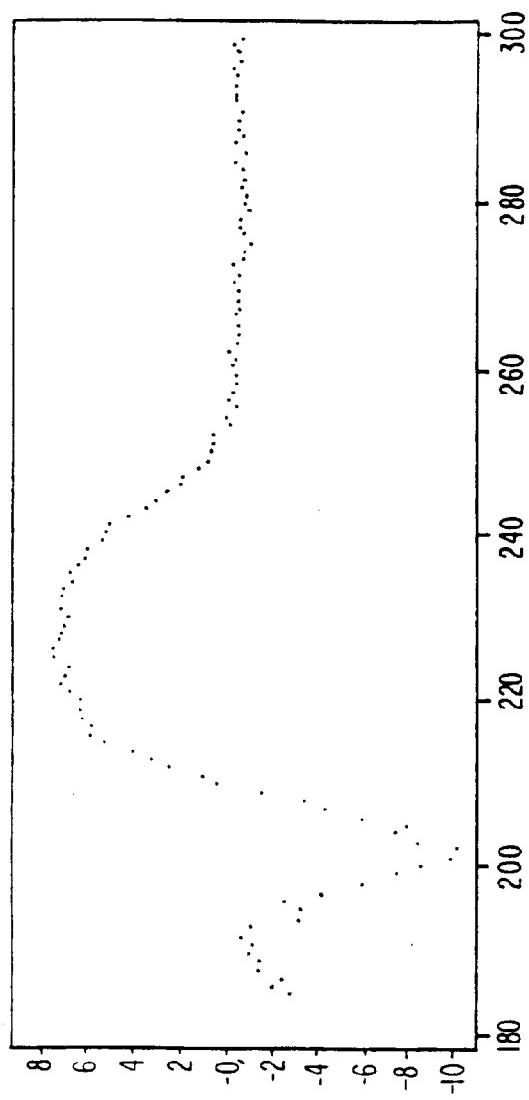


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FIG. 43B

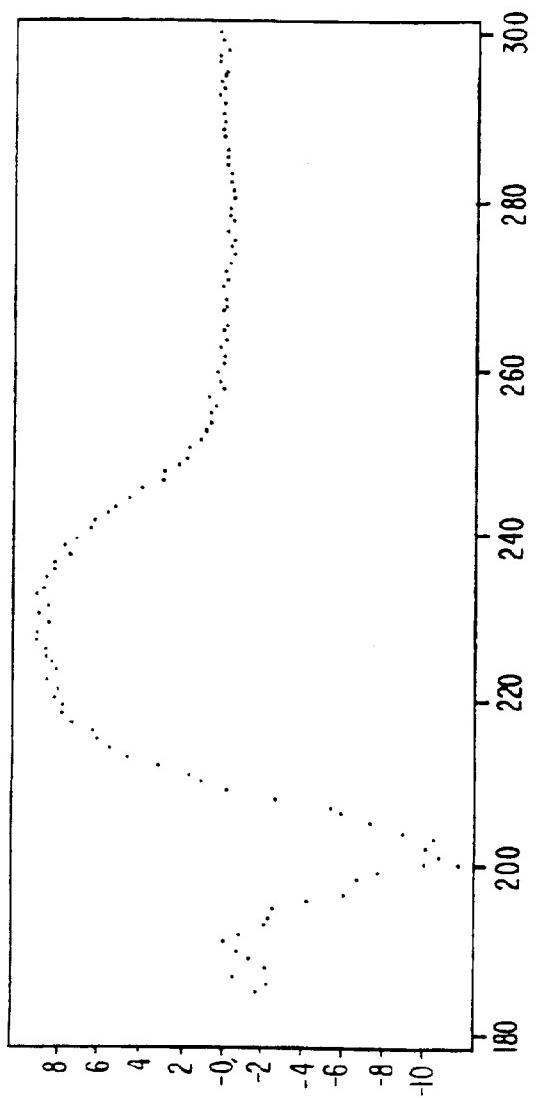
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FIG. 43C



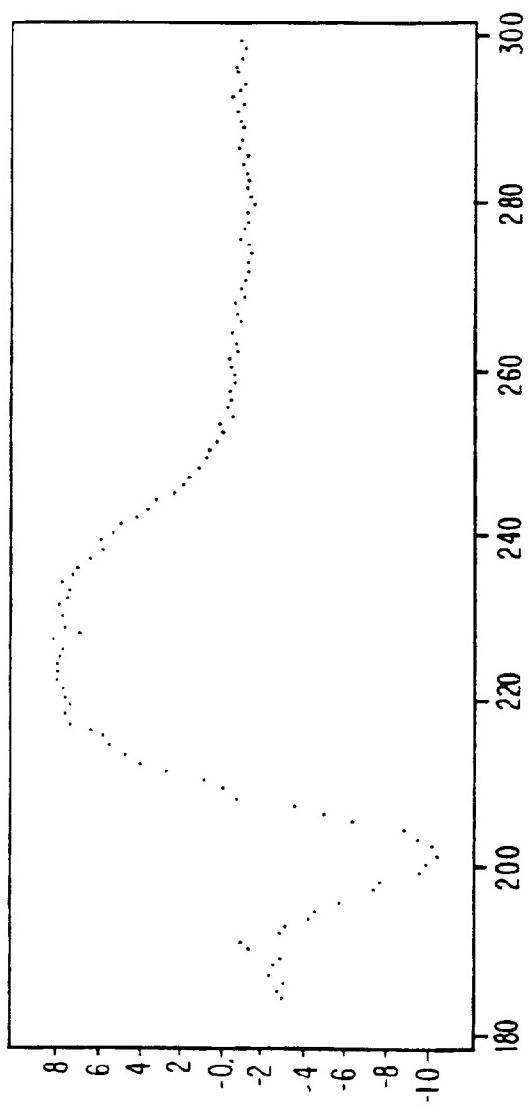
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FIG. 43D



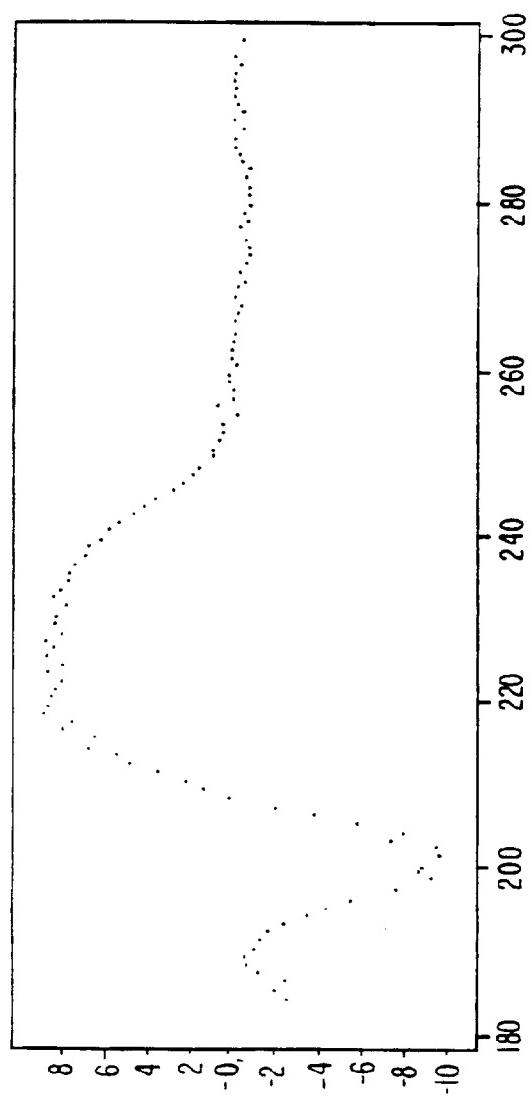
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FIG. 43E



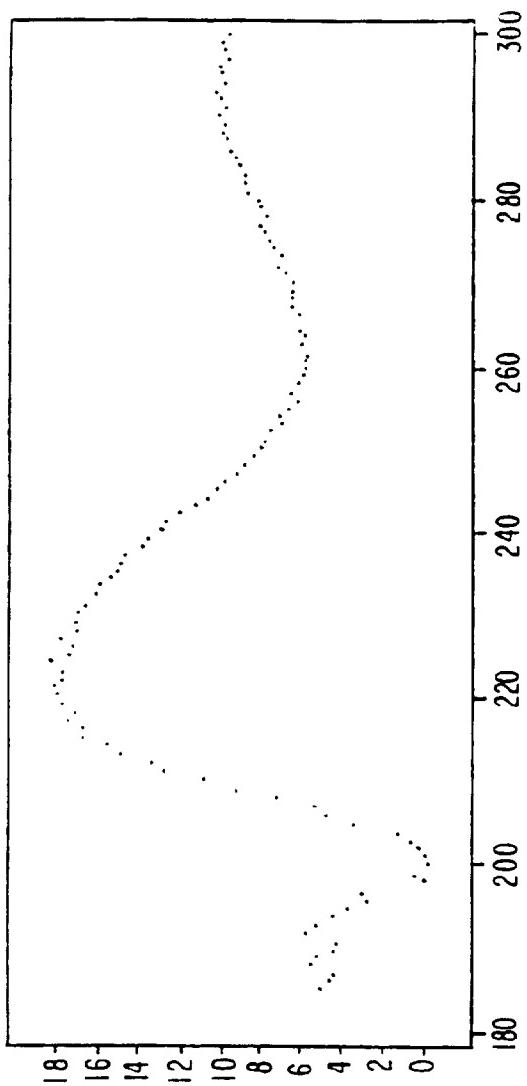
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FIG. 43F

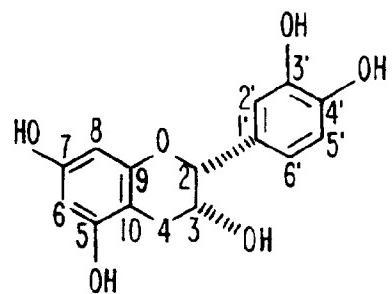


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FIG. 43G



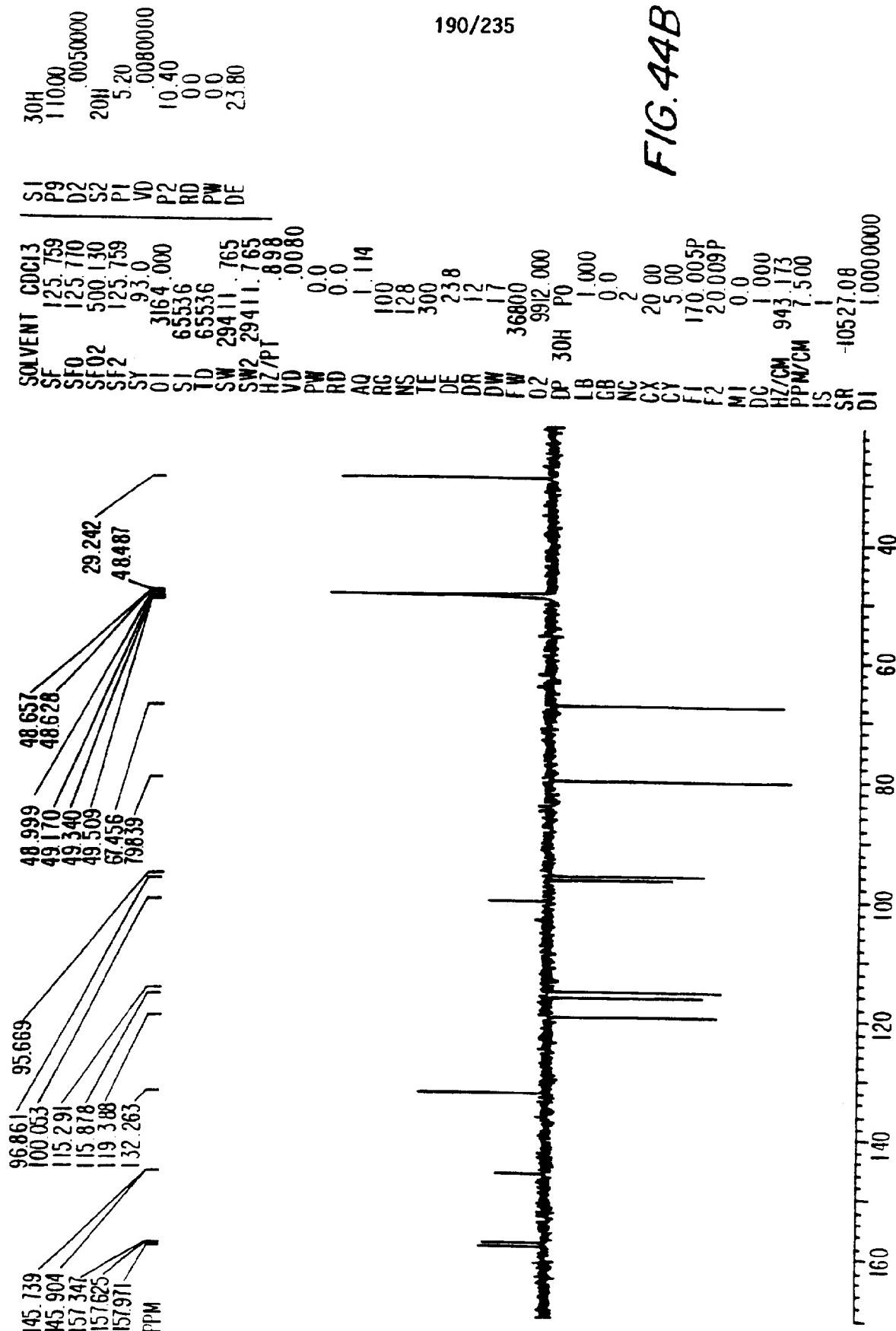
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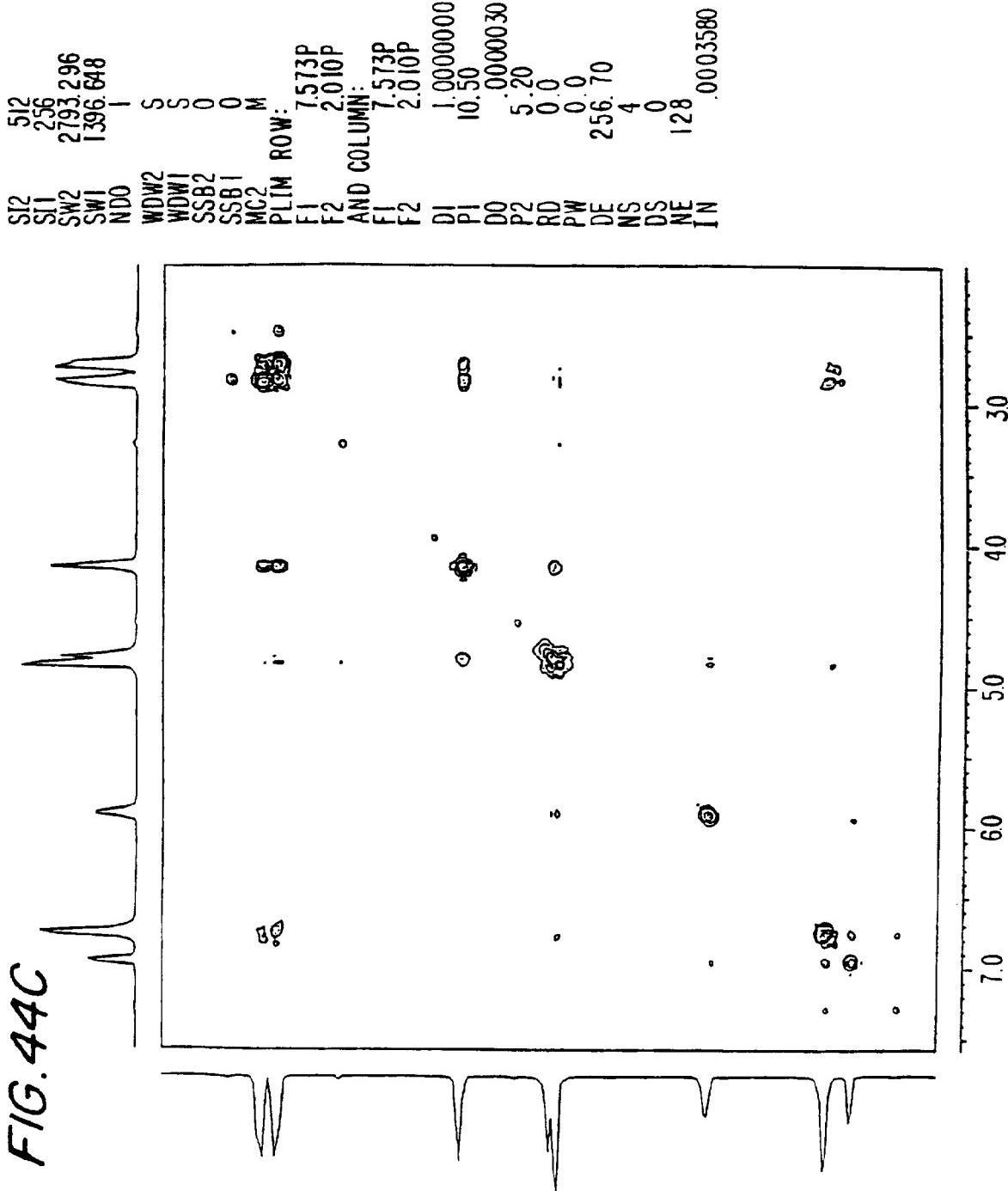
EPICATECHIN			
¹ H	CHEMICAL SHIFT (ppm)	¹³ C	CHEMICAL SHIFT (ppm)
2	4.81	2	79.84
3	4.16	3	67.46
4 _A	{ 2.73	4	29.24
		6	95.87
6	5.94	8	96.36
8	5.91	2'	115.29
5' 6'	{ 6.75 6.79	5' 6'	{ 115.88 119.39
2'	6.97		

FIG. 44A

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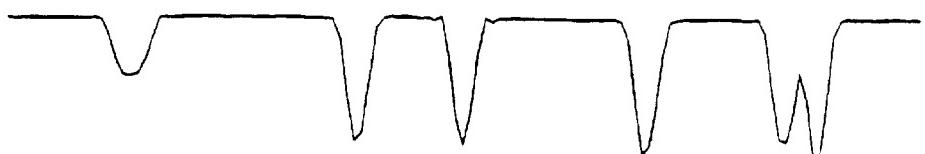
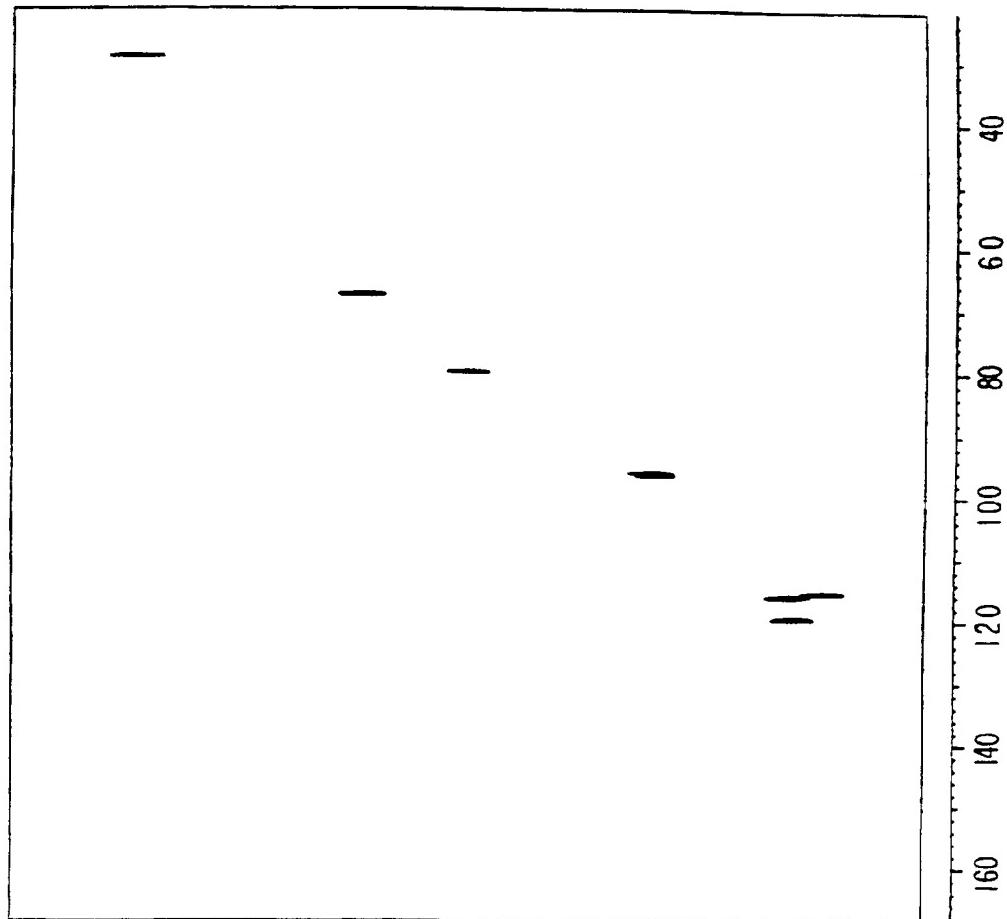
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S2 2048
 S1 28
 SW2 18518519
 SW1 1399776
 ND0 2
 WDW2 S
 WDW1 S
 SSB2 O
 SSB1 O
 MC2 M
 PLIM ROW:
 F1 168.584P
 F2 21.475P
 AND COLUMN:
 F1 21.901P
 F2 -1.87P
 D1 S1 P1 D0 P2 D3 P4 D2 X RD PW DE DS DS P9 NE IN
 OH 10.50 0.0000030 1.040 5.20 0.0040000 20H 0.0 0.0 40.80 64 110.00 64 .0001786

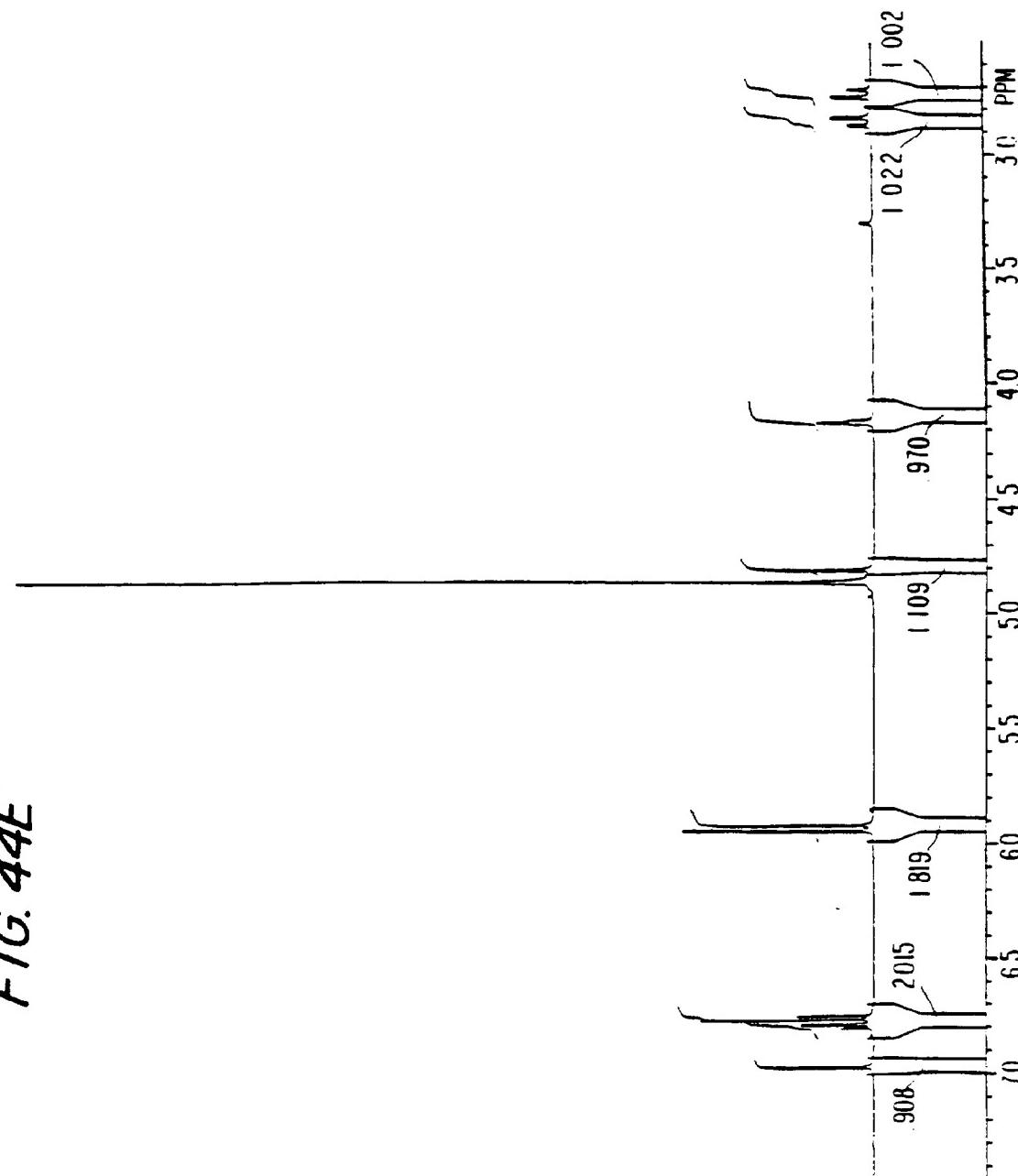
FIG. 44D



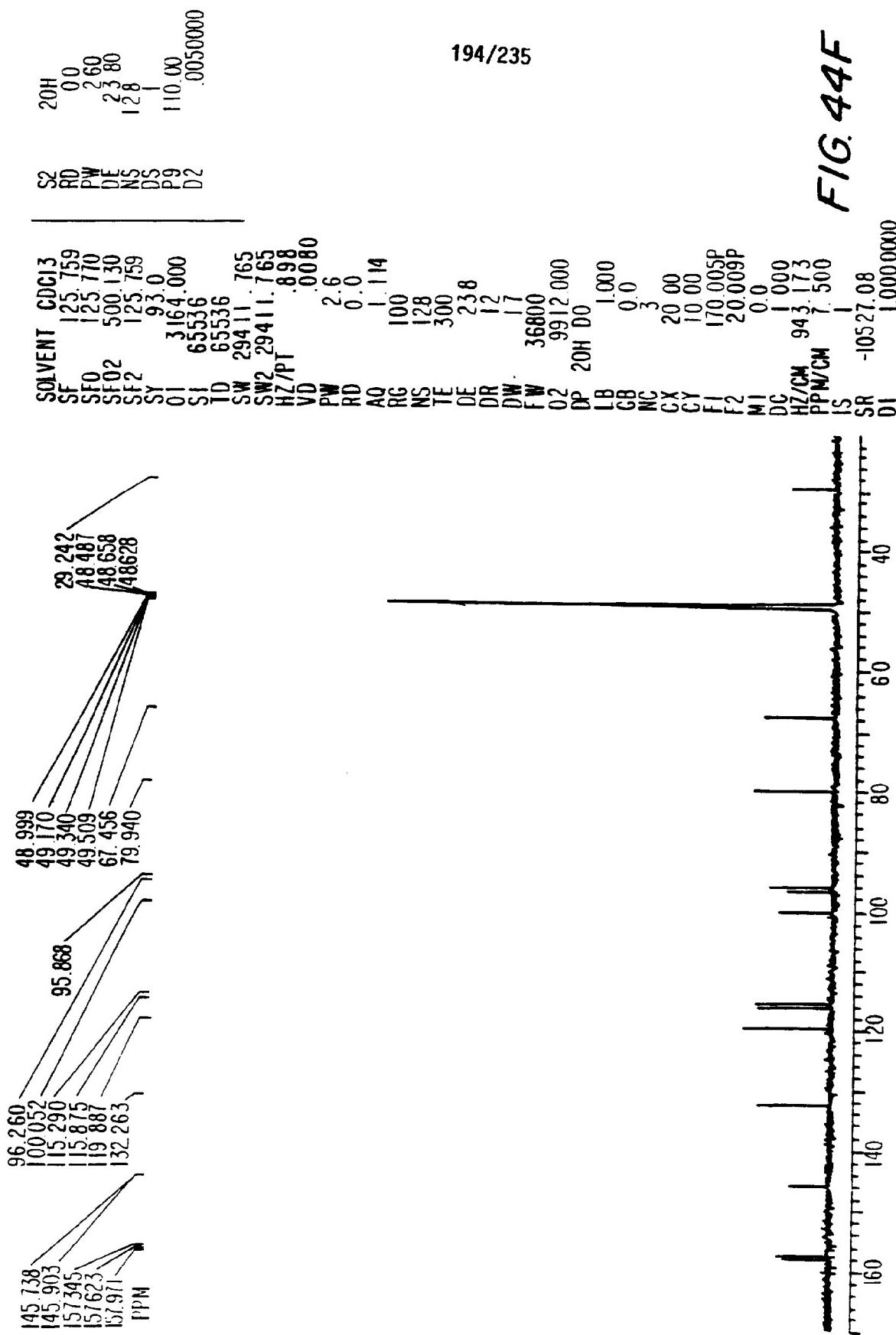
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SOLVENT	CDCl ₃
SF	500.137
SF0	500.130
SF02	500.130
SF2	500.137
SY	1.66.0.90
SI	9894.5.90
TD	32768
SW	32768
SW2	6024.096
HZ/PT	6024.096
VD	368
PW	0.0
RD	0.0
AQ	2.720
RG	8
NS	16
TE	300
DE	106.3
DR	12
DW	83
FW	7600
O2	50000.000
DP	63LP0
LB	0.0
GB	0.0
NC	0
CX	2000
CY	1500
F1	7500P
F2	2500P
M1	0.0
DC	1.000
HZ/CM	125.030
PPM/CM	.250
IS	3
SR	7394.06

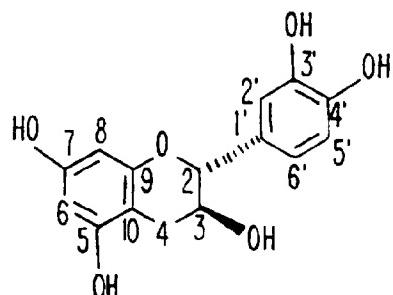
FIG. 44E



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CATECHIN			
^1H	CHEMICAL SHIFT (ppm)	^{13}C	CHEMICAL SHIFT (ppm)
2	4.56	2	79.84
3	3.97	3	67.46
4α	2.50	4	29.24
4β	2.84	6	95.87
6	5.85	8	96.36
8	5.92	2'	115.29
2'	6.83	6' 5'	116.08 120.08
5'	6.76		
6'	6.71		

FIG.45A

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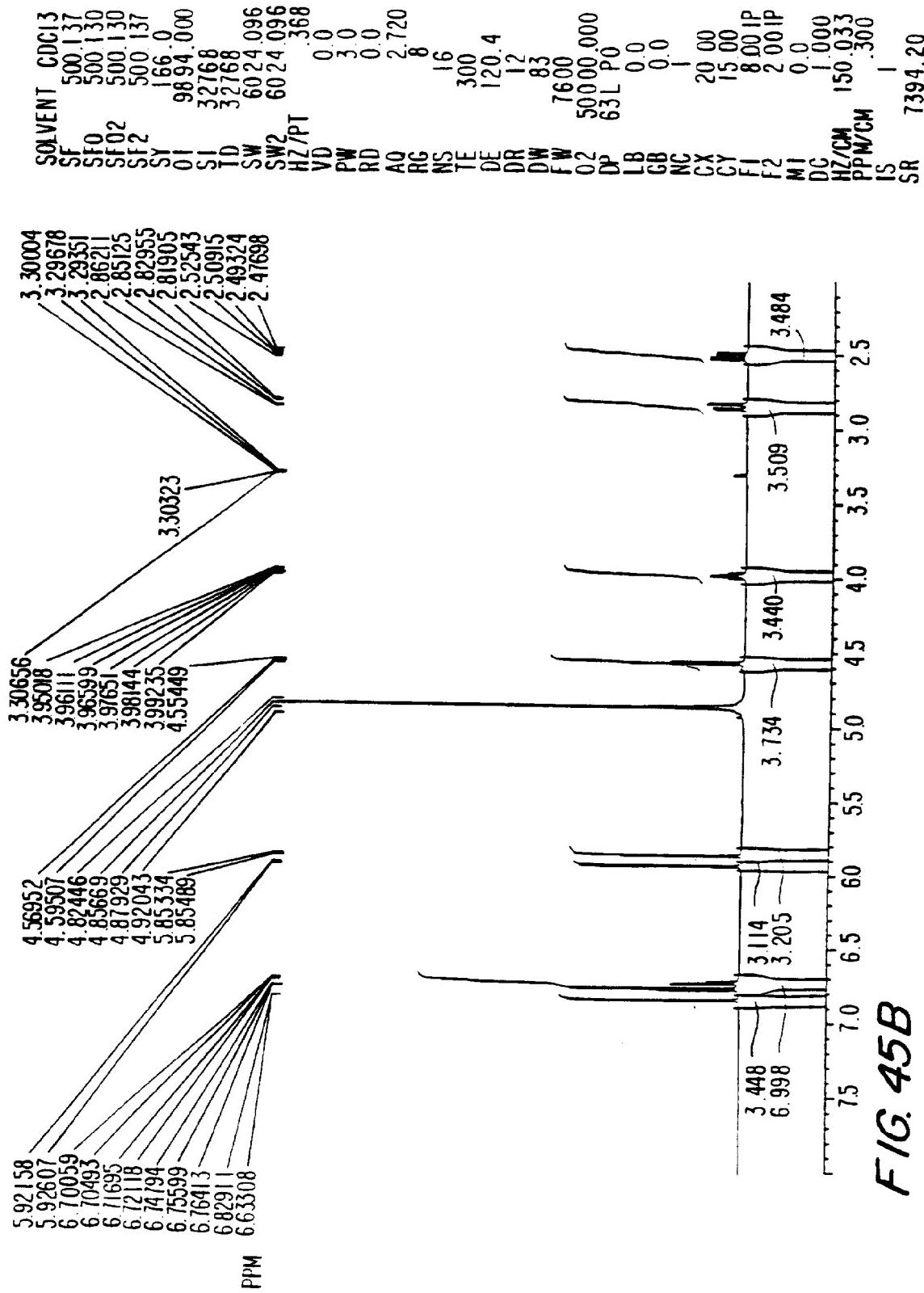
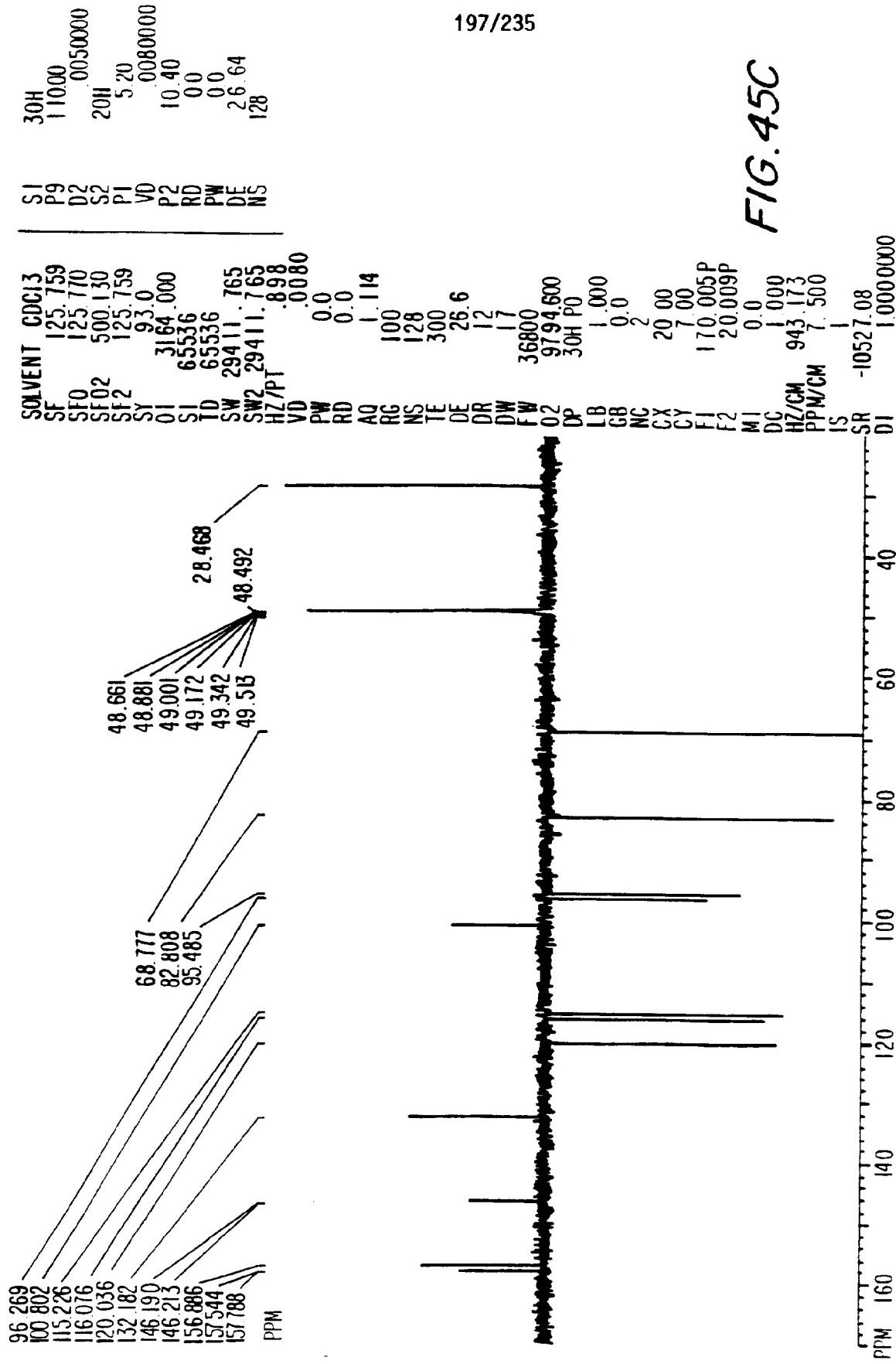
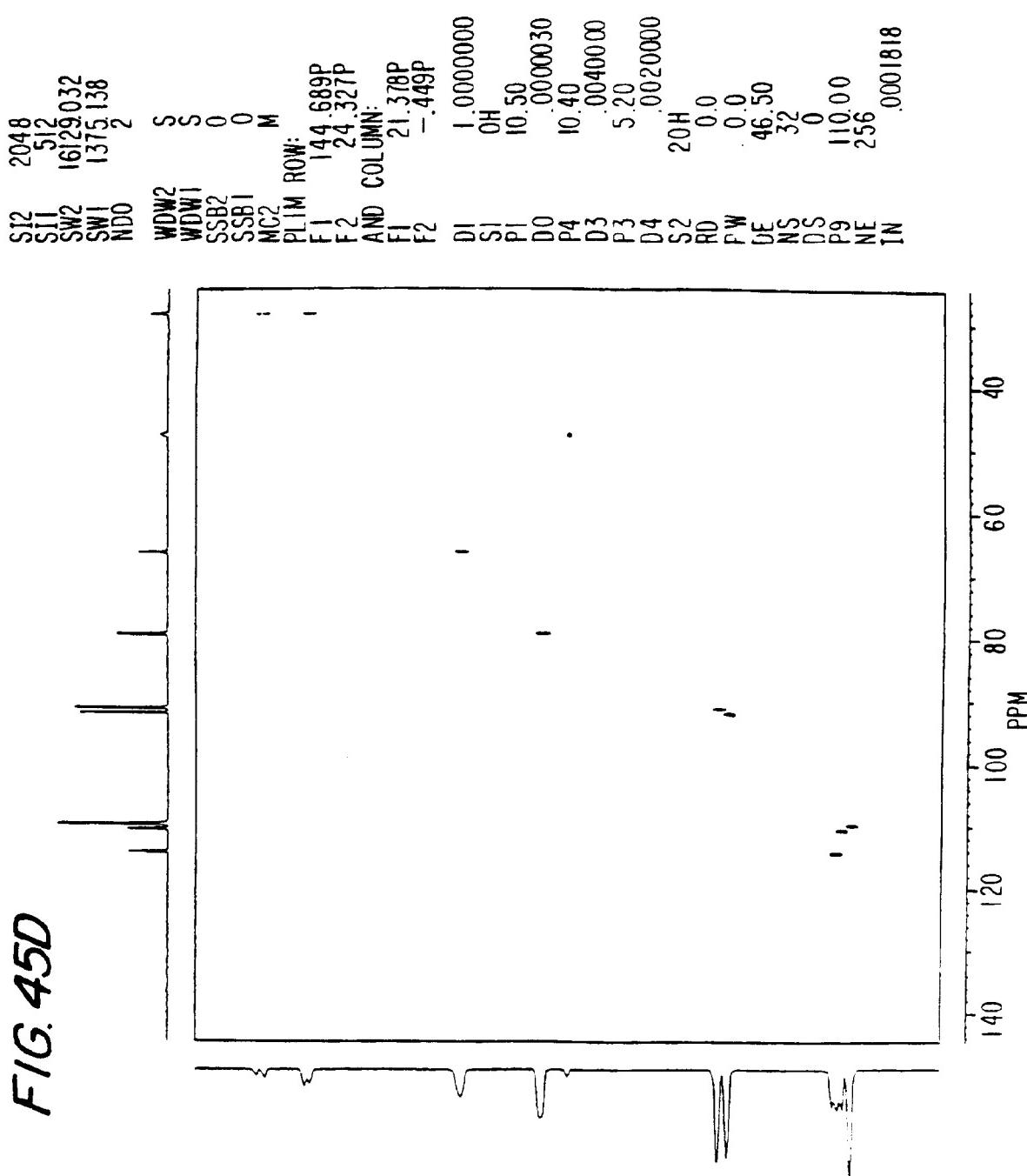


FIG. 45B

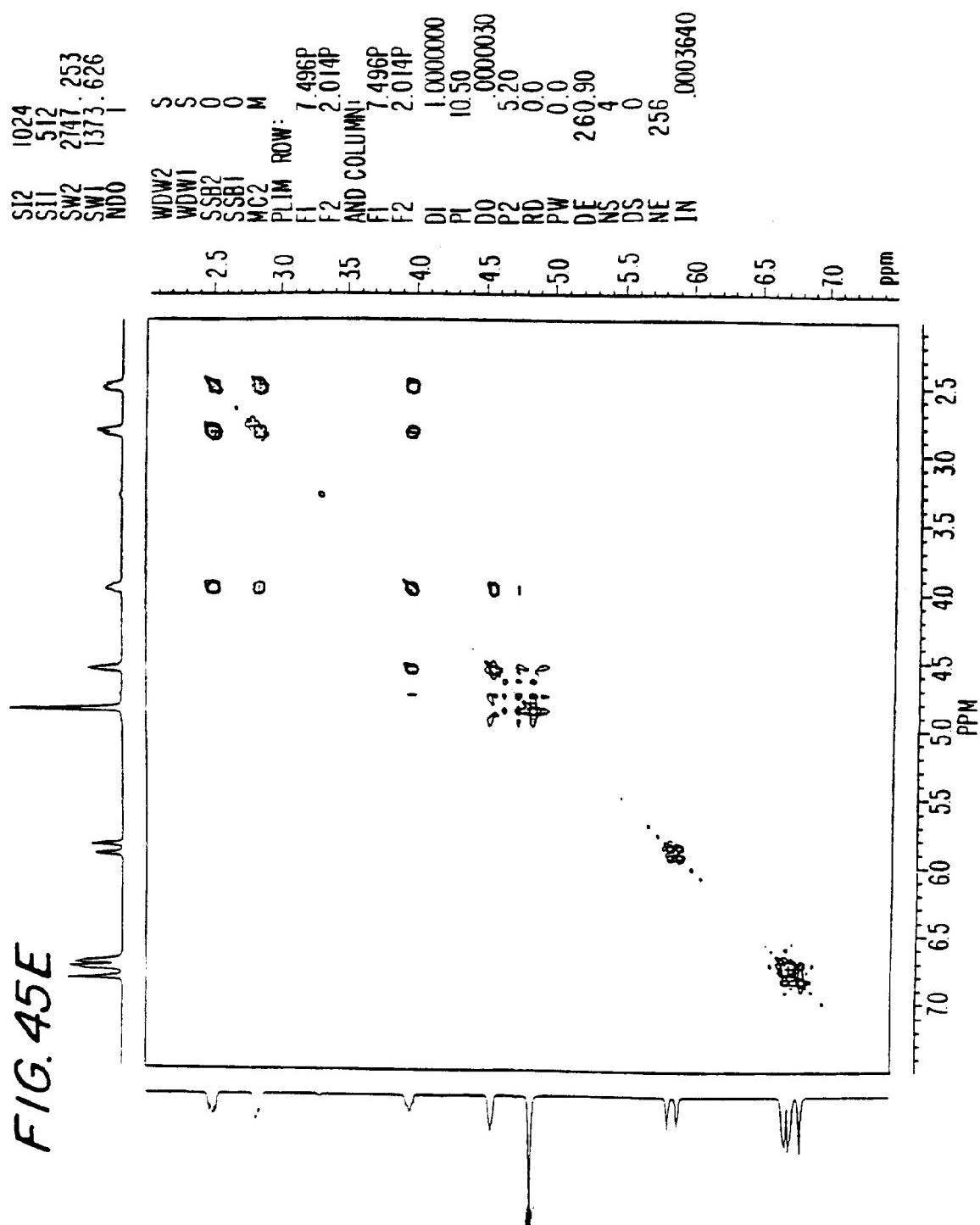
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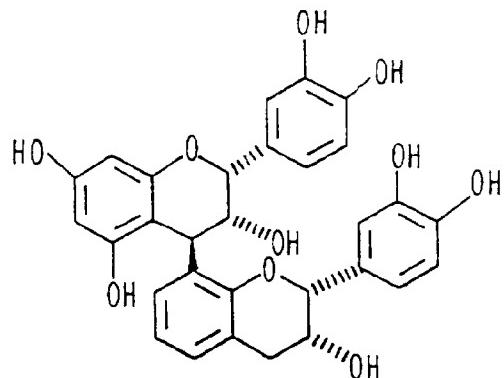


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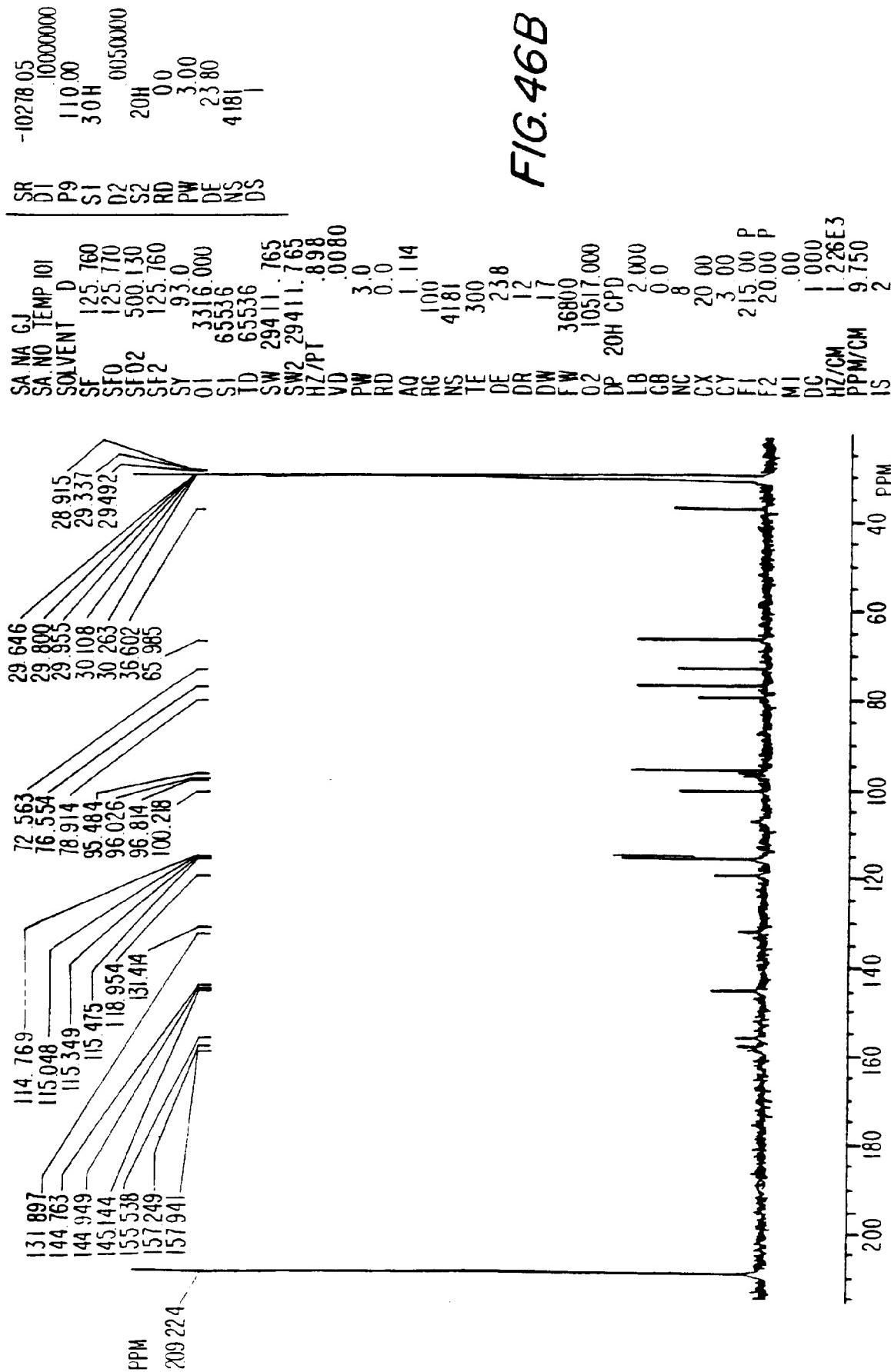
SUBSTITUTE SHEET (RULE 26)

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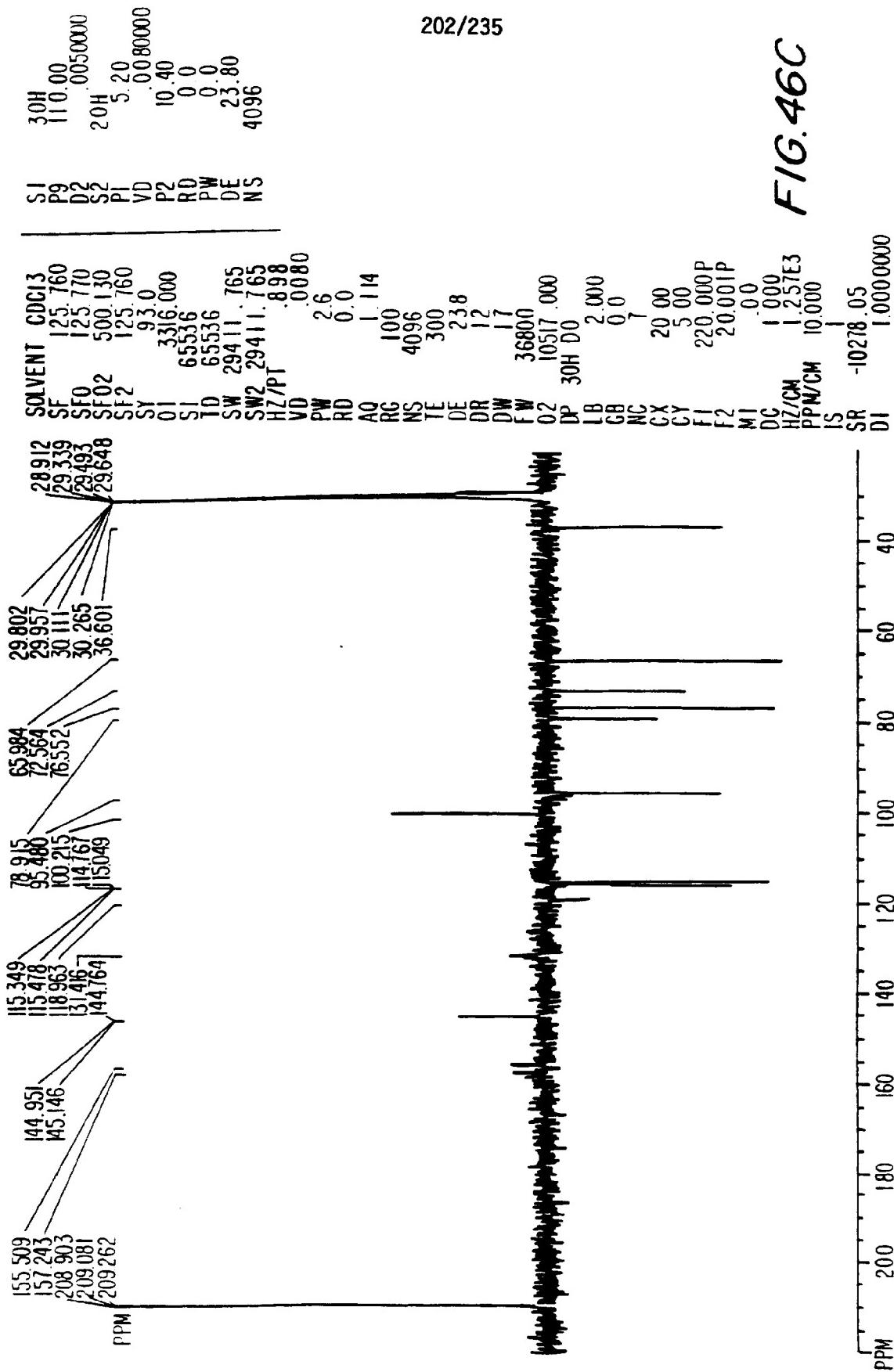


B2 DIMER			
¹ H	CHEMICAL SHIFT (ppm)	¹³ C	CHEMICAL SHIFT (ppm)
B4	2.69 2.83	B4 T4	28.92 36.60
T4	4.63	B3	65.99
B3	4.29	T3	72.56
T3	3.85	T2	76.55
T2	4.99	B2	78.91
B2	4.92	T6 OR 8	95.48
B6	5.92	B6	96.03
T6 OR 8	5.91	T6 OR 8	96.81
T6 OR 8	5.98		100.22
B2'	7.12	B2'	114.77
T2'	6.92	T2'	115.05
T5	6.68	T5'	115.35
B5	6.70	B5'	115.48
T6'	6.87	T6'	118.95
B6'	6.58	B6'	
		T1'	131.41 131.90

FIG. 46A



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SUBSTITUTE SHEET (RULE 26)

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F2-ACQUISITION PARAMETERS

PULPROG	79pr
SOLVENT	AQ
TDRES	3.76833384
DW	0.132685
RG	115.0
NUCLEUS	¹ H
HL1	
DL12	0.0000200
HL2	76
PI8	1000000.0
DI3	0.0000040
P1	5.0
DE	164.3
SFO1	360.1373784
SWH	4347.83
TD	32768
NS	16
DS	0

F2- PROCESSING PARAMETERS

SI	163.84
SF	360.1358580

WDW	no
SSB	0
LB	0.00
GB	0

PC	1.00
CX	22.00

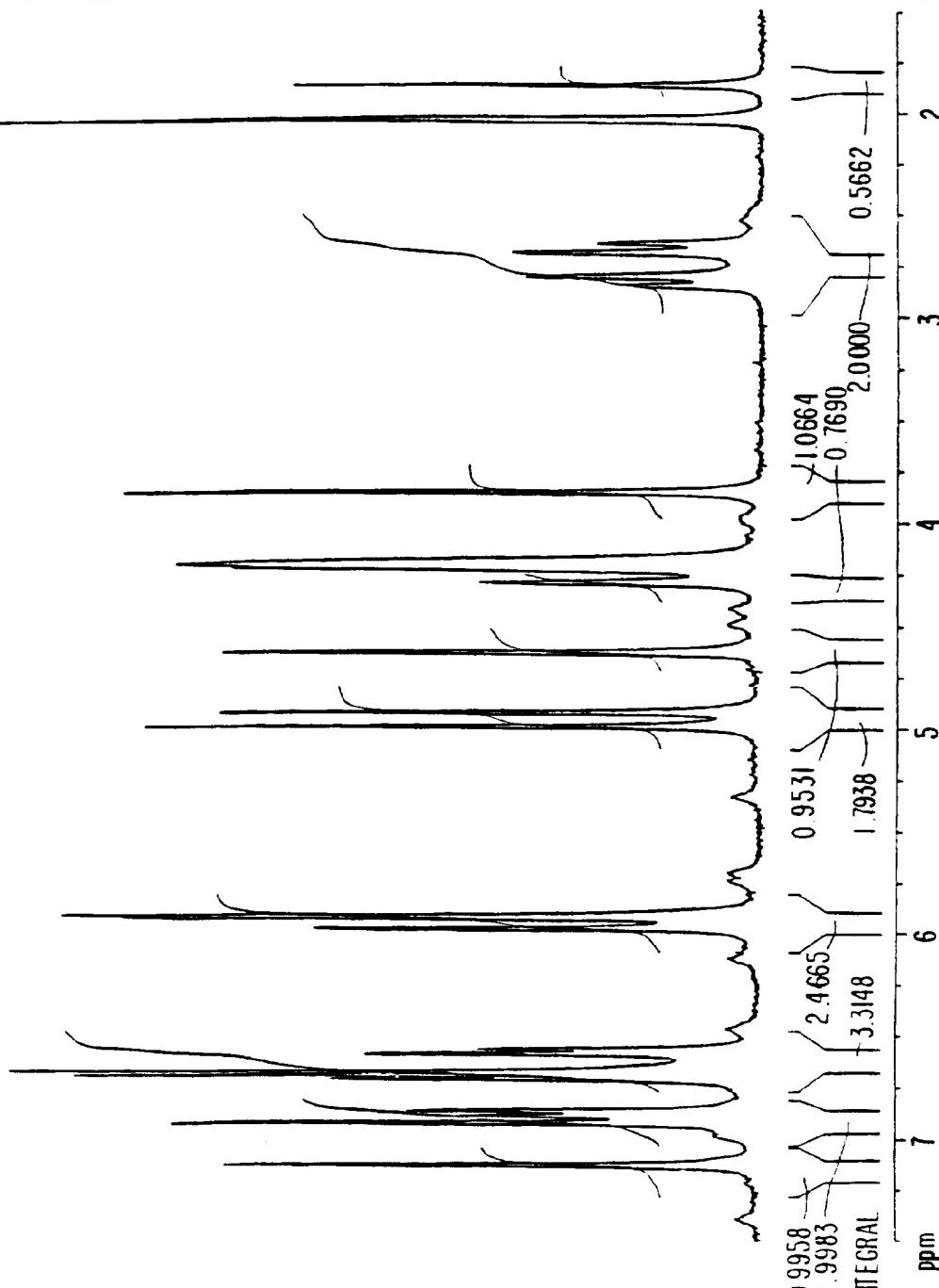
FIP	7.500
FI	2701.02

F2P	1.500
F2	540.20

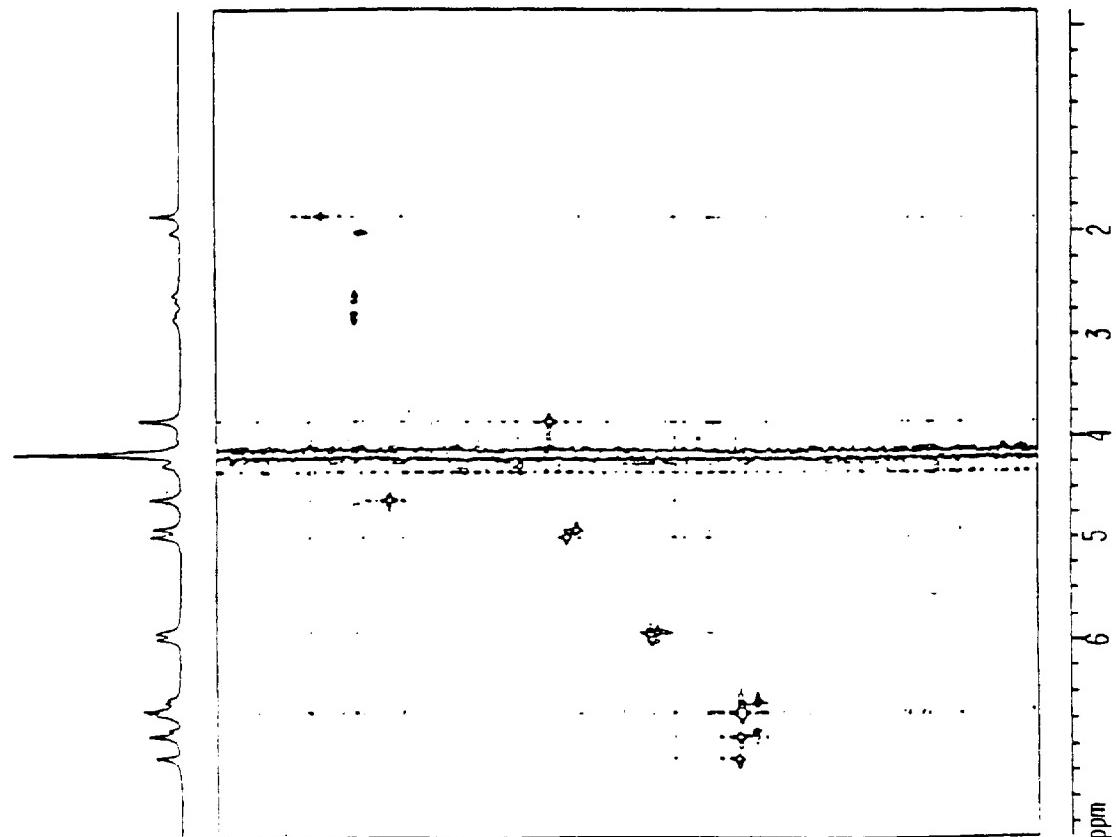
PPMCM	0.27273
HZCM	98.21887

FIG. 46D

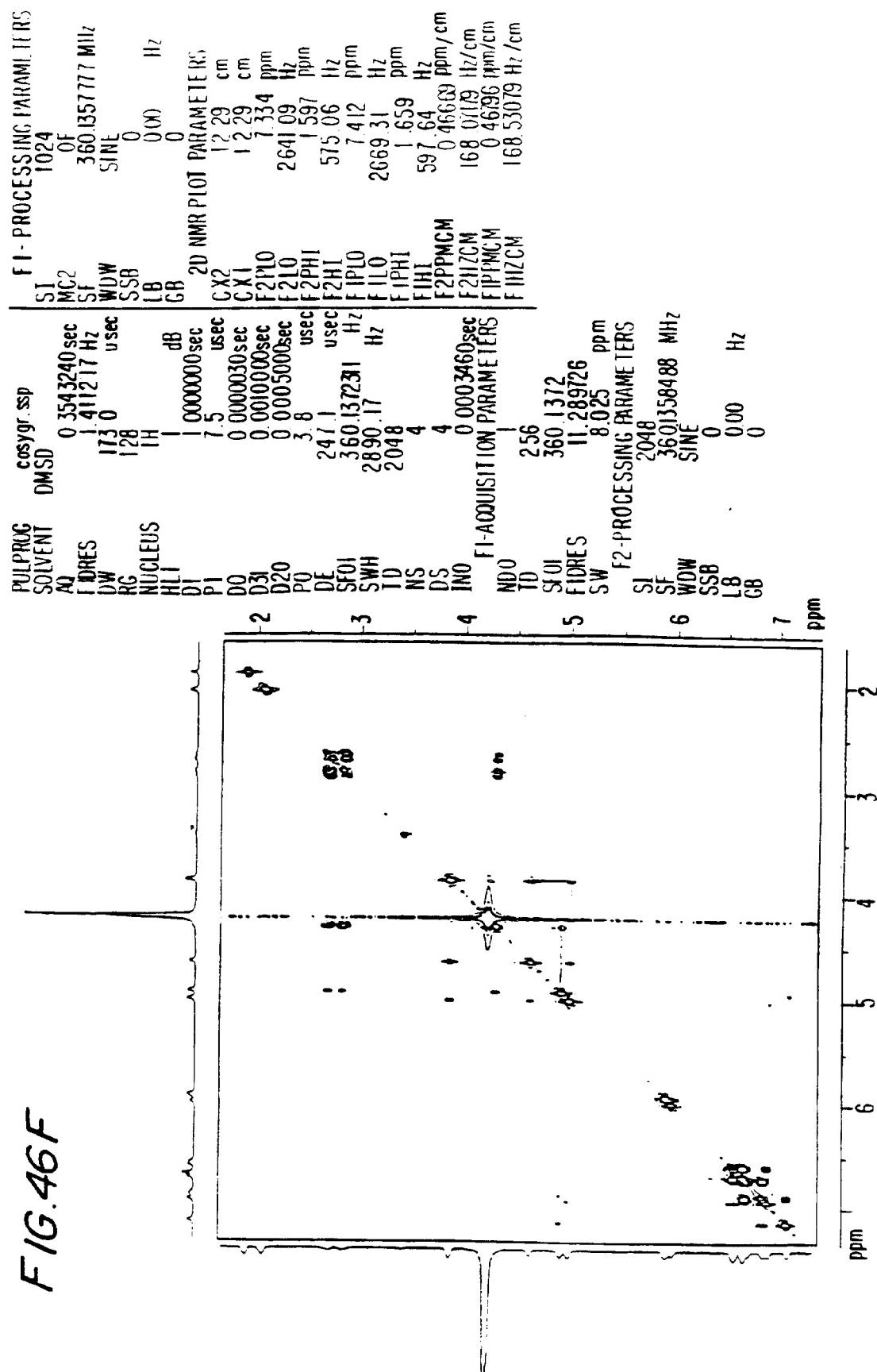
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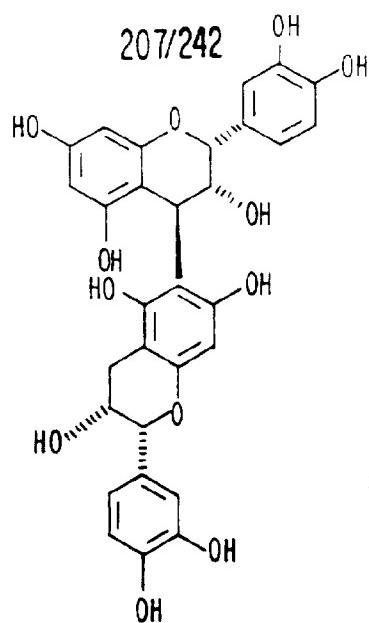
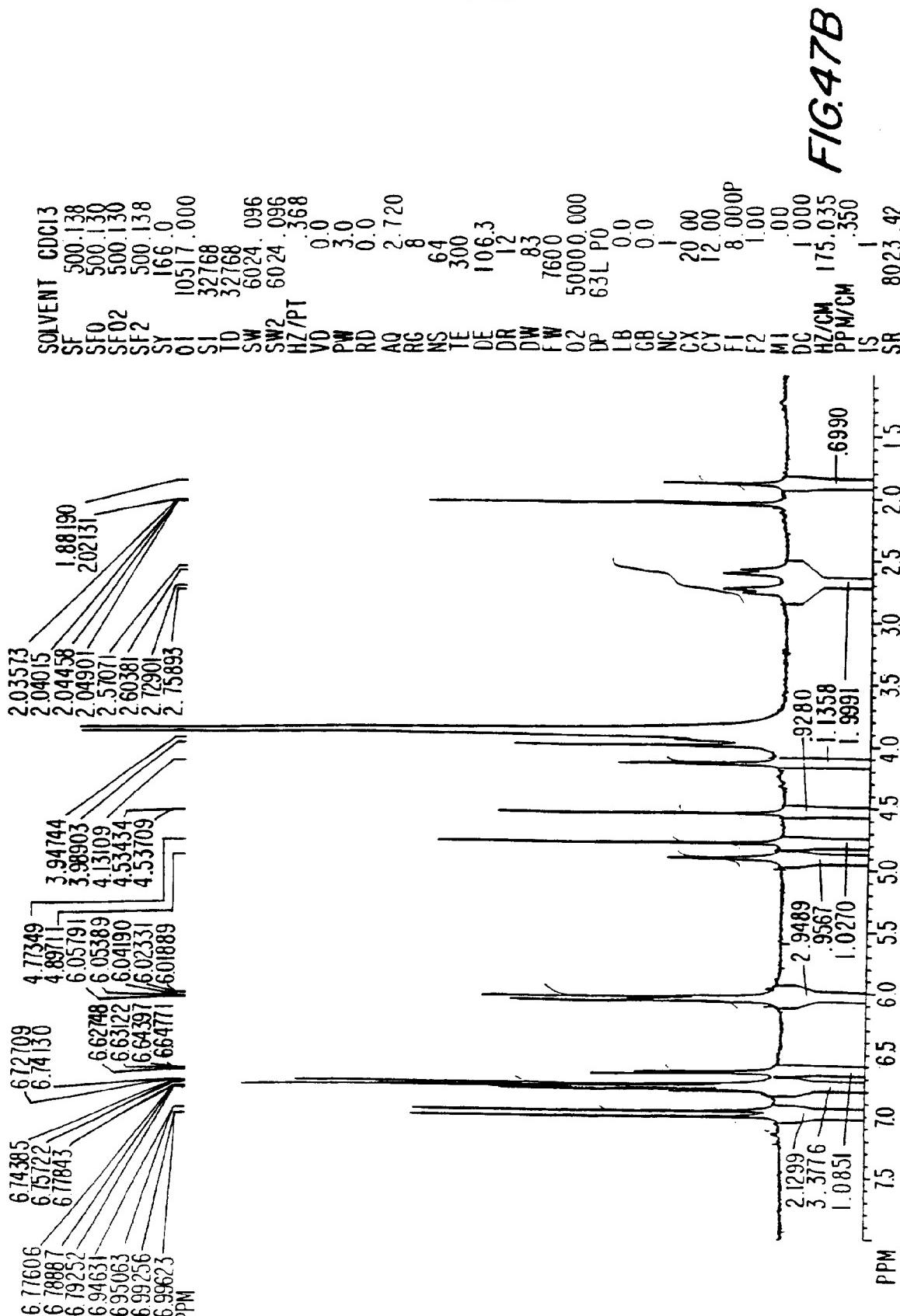


FIG. 47A

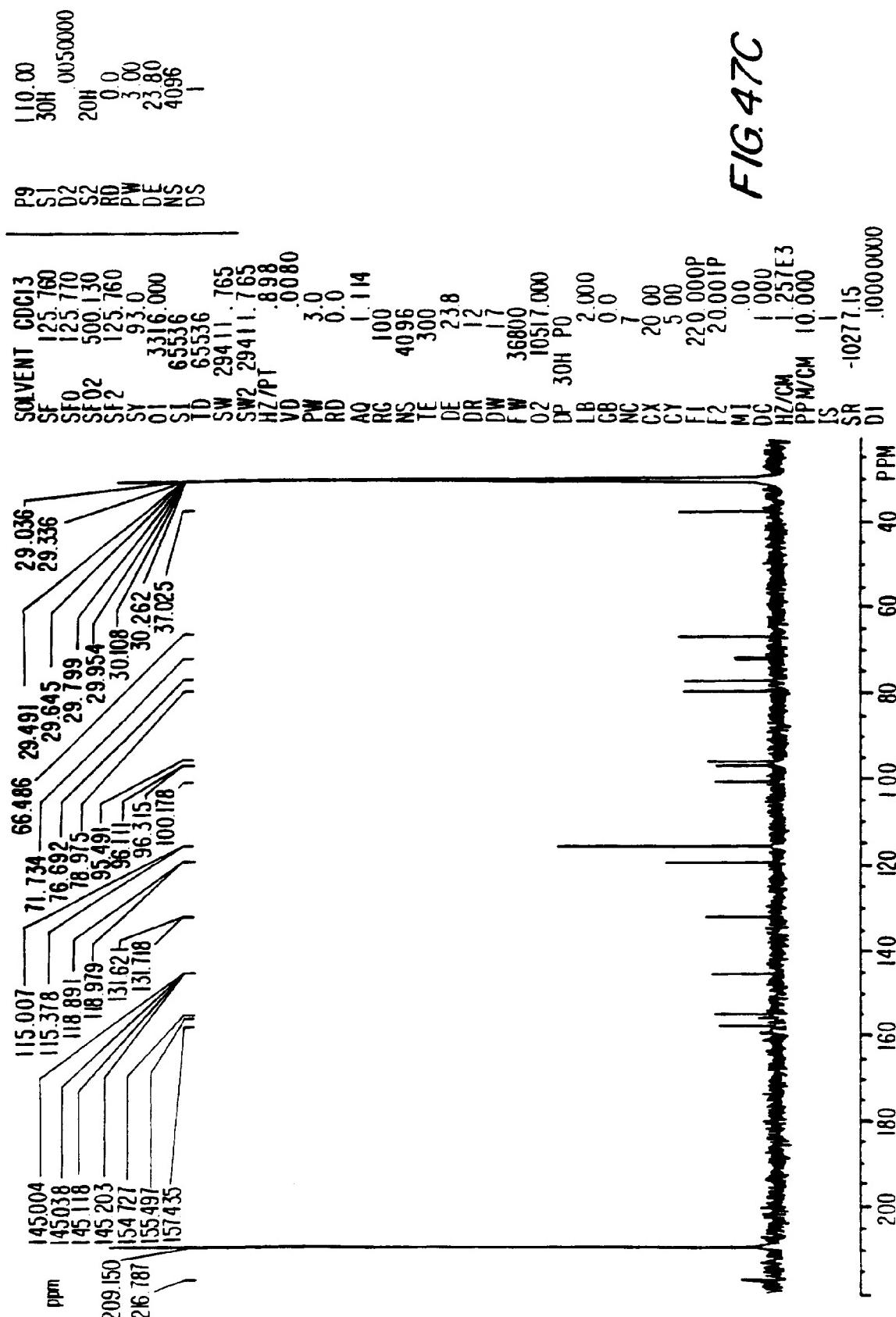
B5 DIMER			
δ_{H}	CHEMICAL SHIFT (ppm)	δ_{C}	CHEMICAL SHIFT (ppm)
B4	2.585 7.745	B4 T4	29.04 37.03
T3	3.99	B3	66.49
B3	4.13	T3	71.73
T4	4.54	T2	76.69
B2	4.77	B2	78.98
T2	4.90	B6+8	95.49
T6+	6.02		96.11
T8	6.055	T6+8	96.32
B8	6.04		100.18
T6'	6.64	T2+5	115.01
T5'	6.74	B2'+5'	115.38
B5'	6.75	B6	118.90
B6'	6.78	T6	118.98
T2'	6.95	B1'	131.62
B2'	6.99	T1'	131.72
		B & T	145.00
		3' + 4'	145.04 145.04 145.12 145.20
		B + T 5, 7 + 8a	154.73 155.50 157.44

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SUBSTITUTE SHEET (RULE 26)

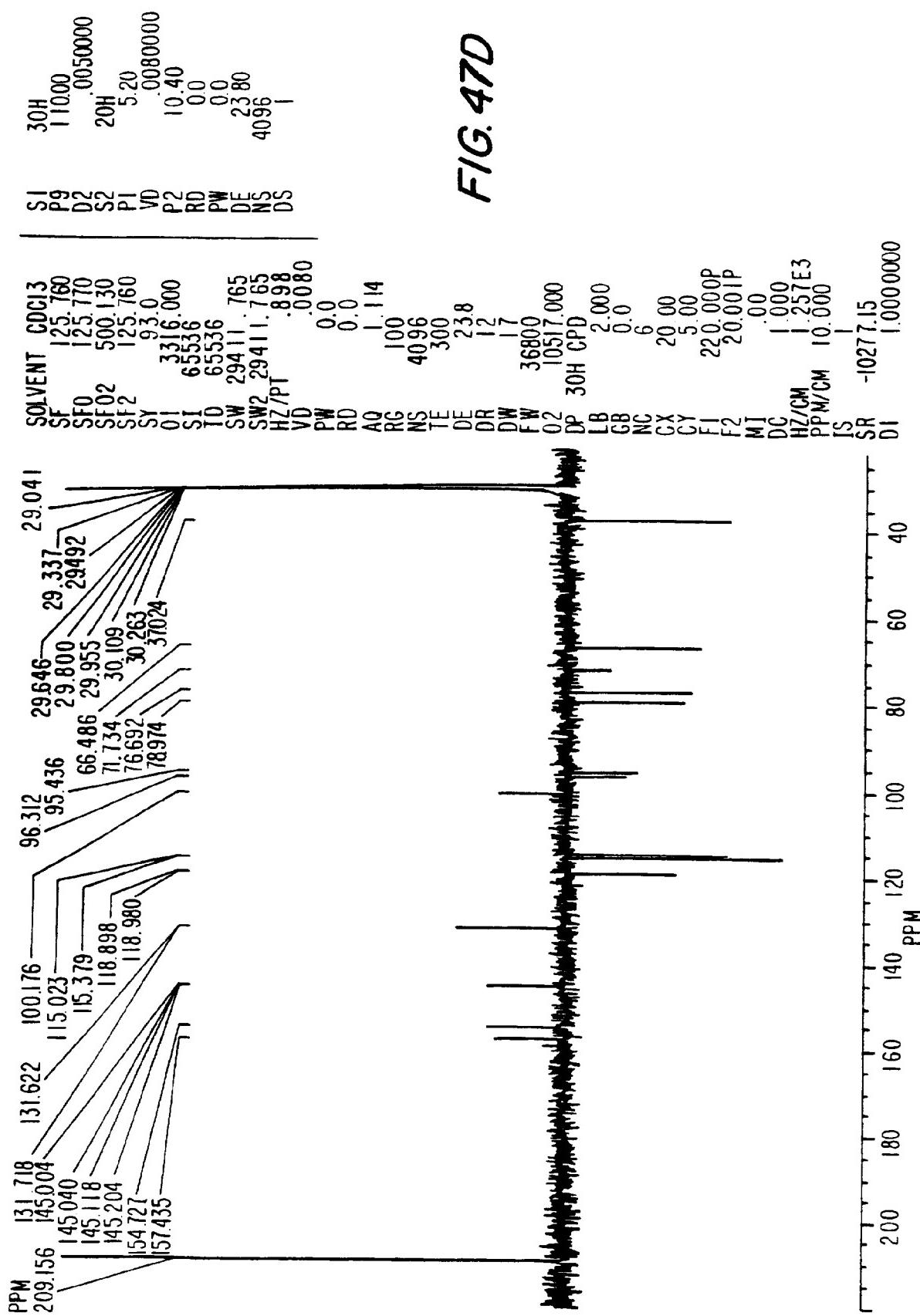
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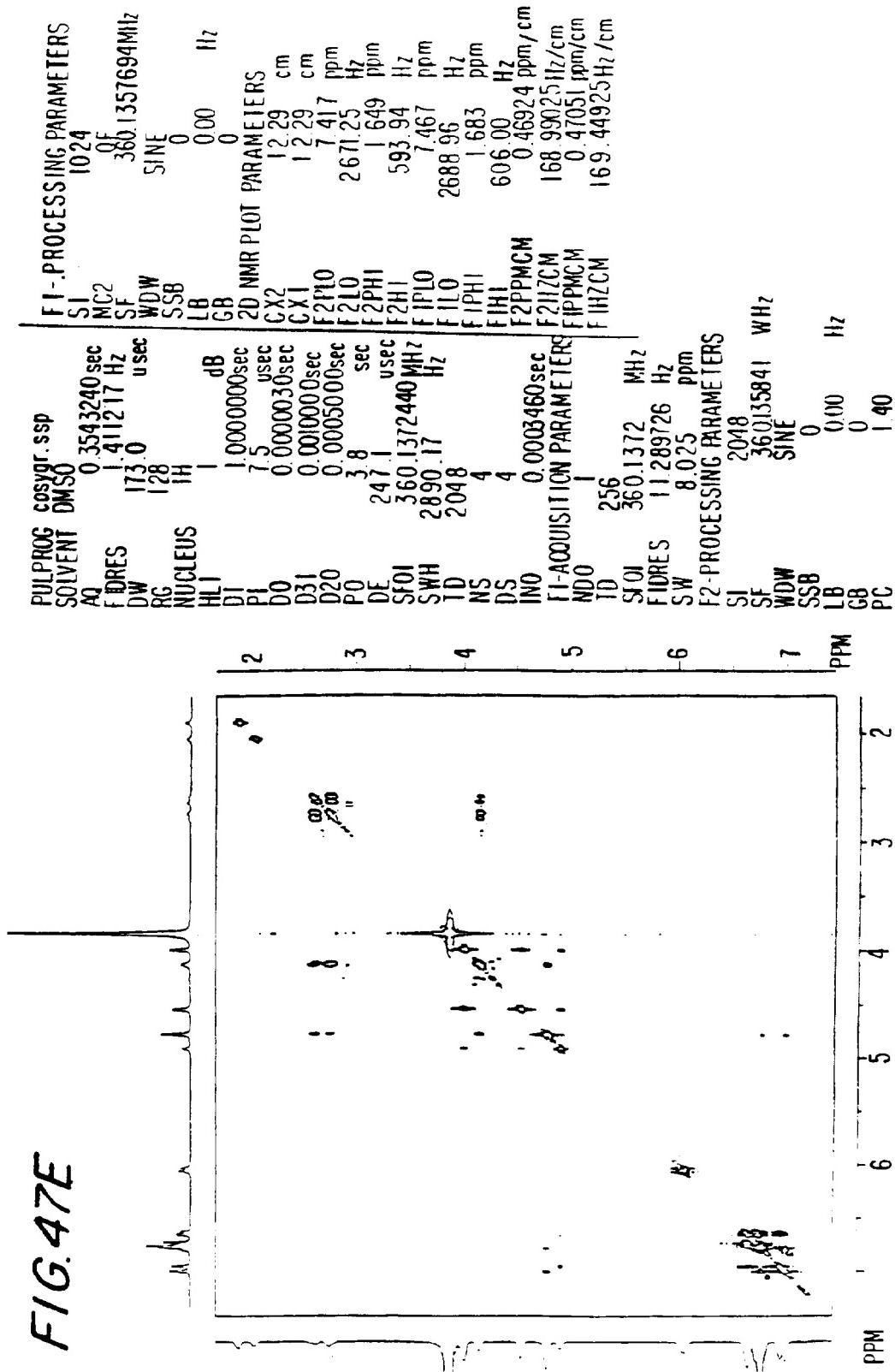


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FIG. 47D





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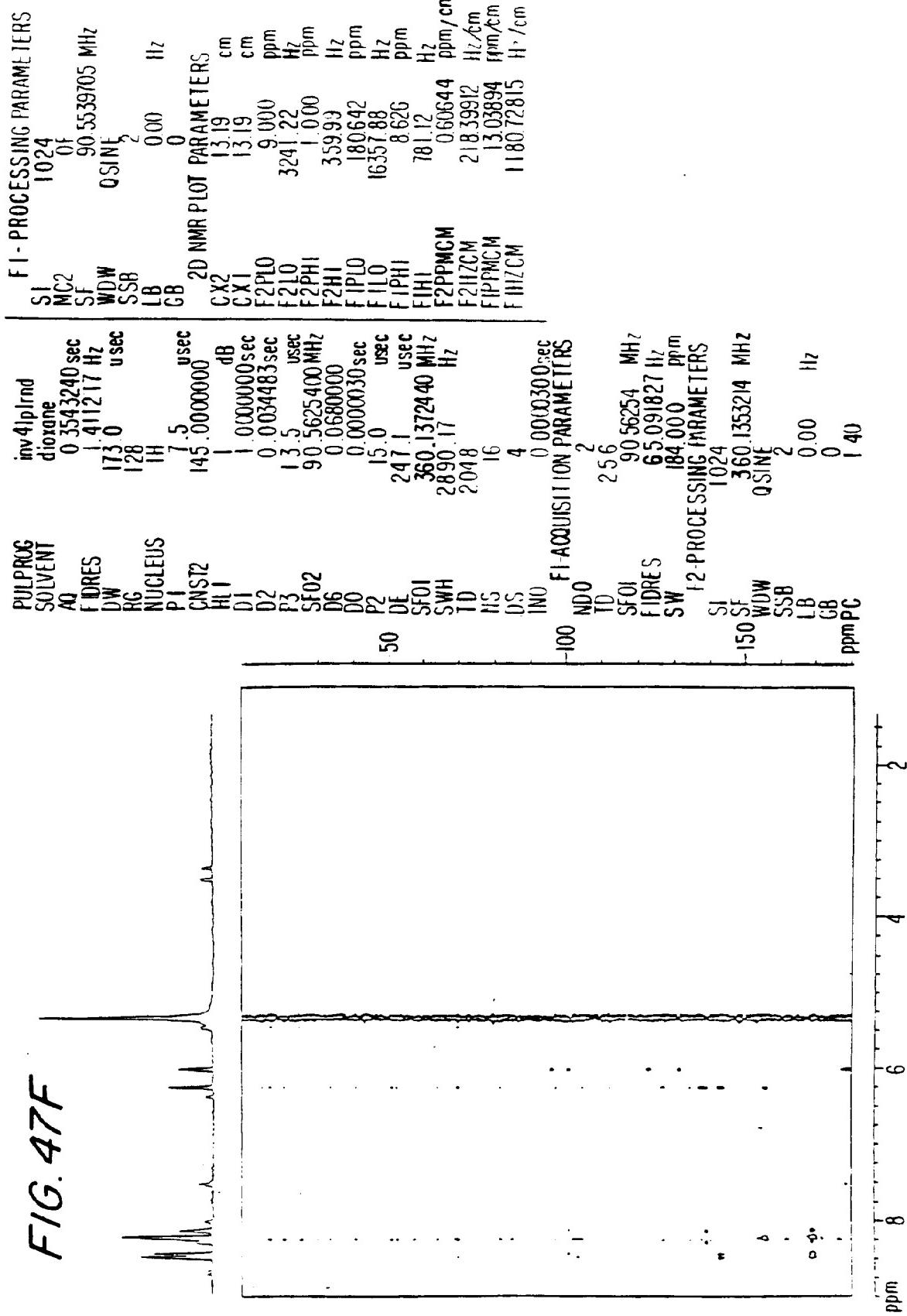
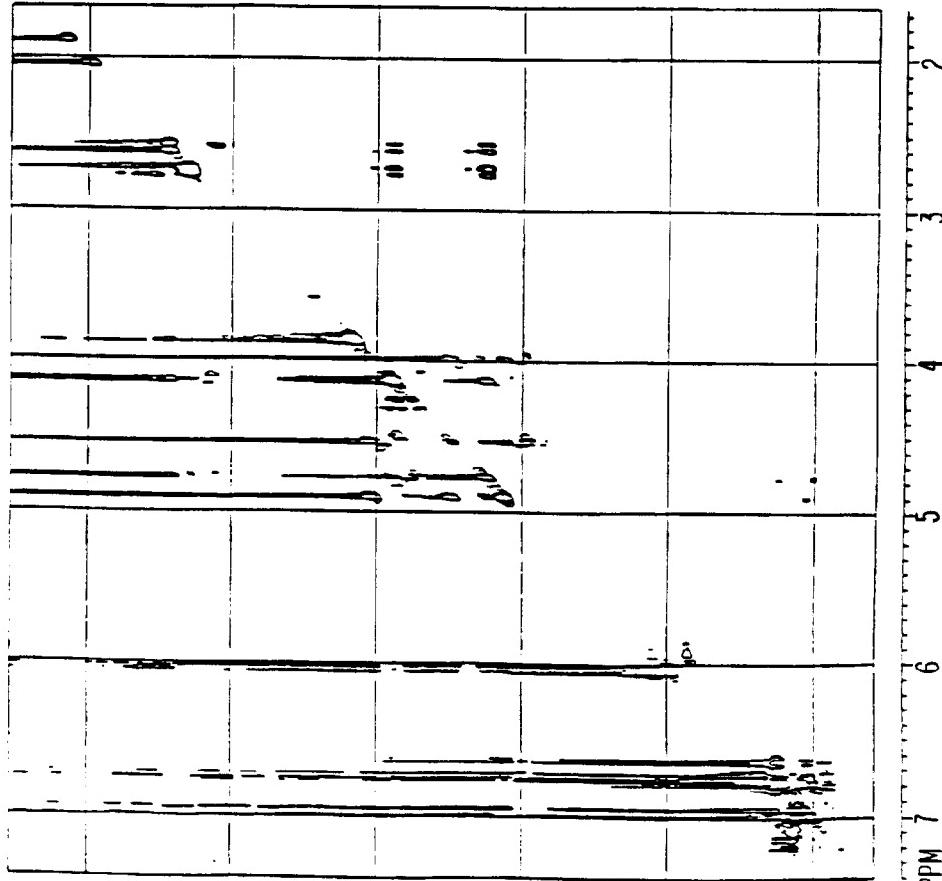
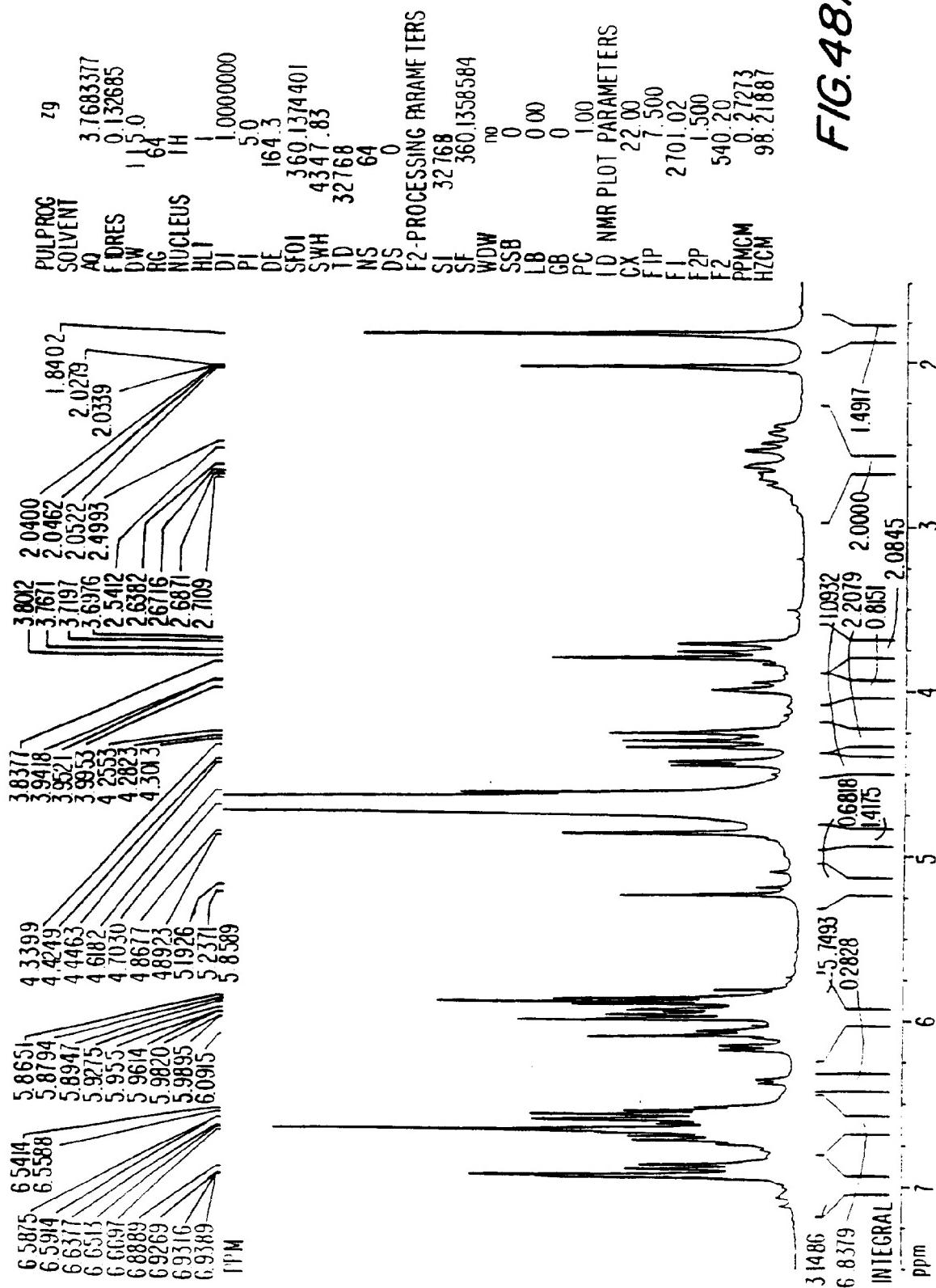


FIG. 47F

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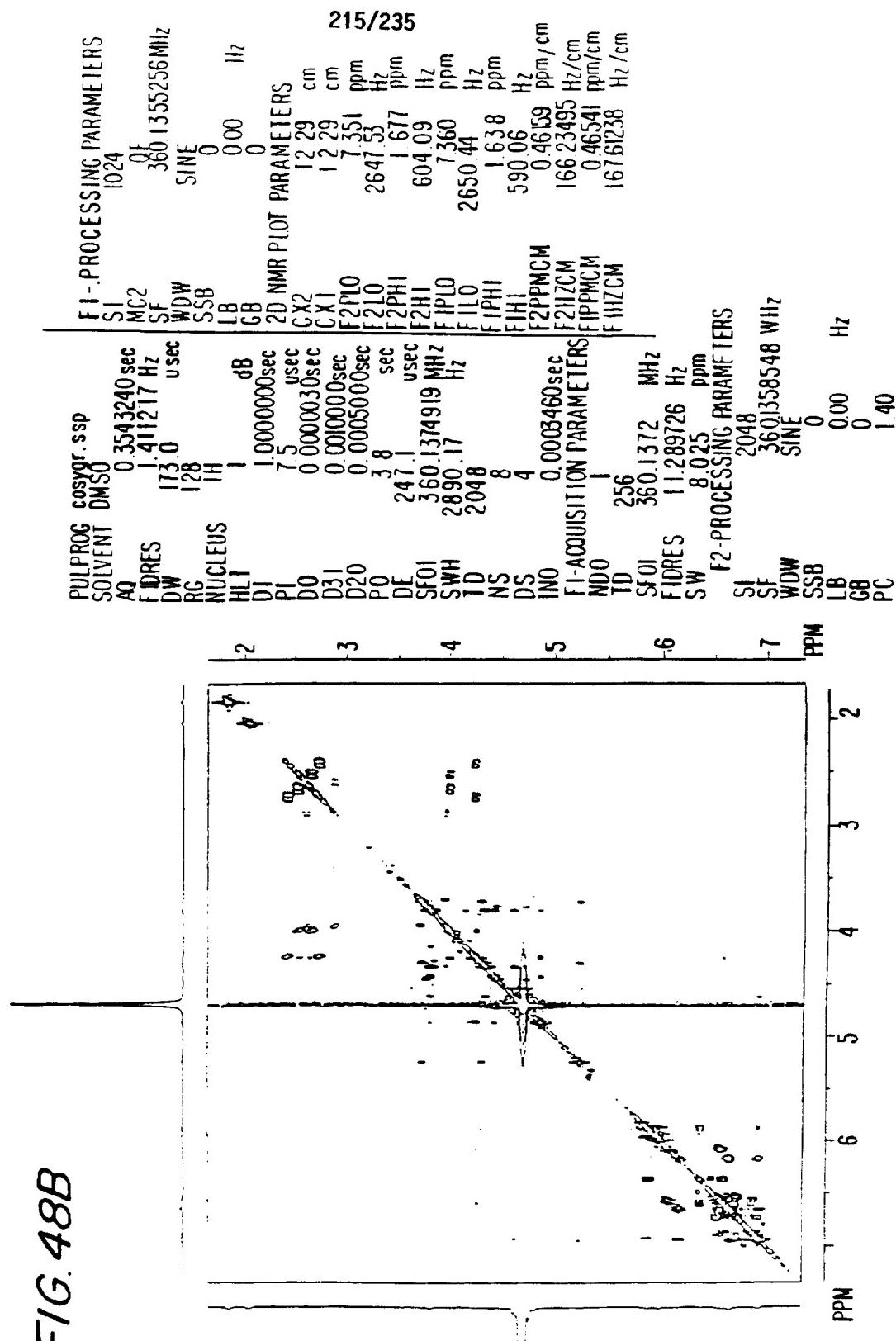


FIG. 48B

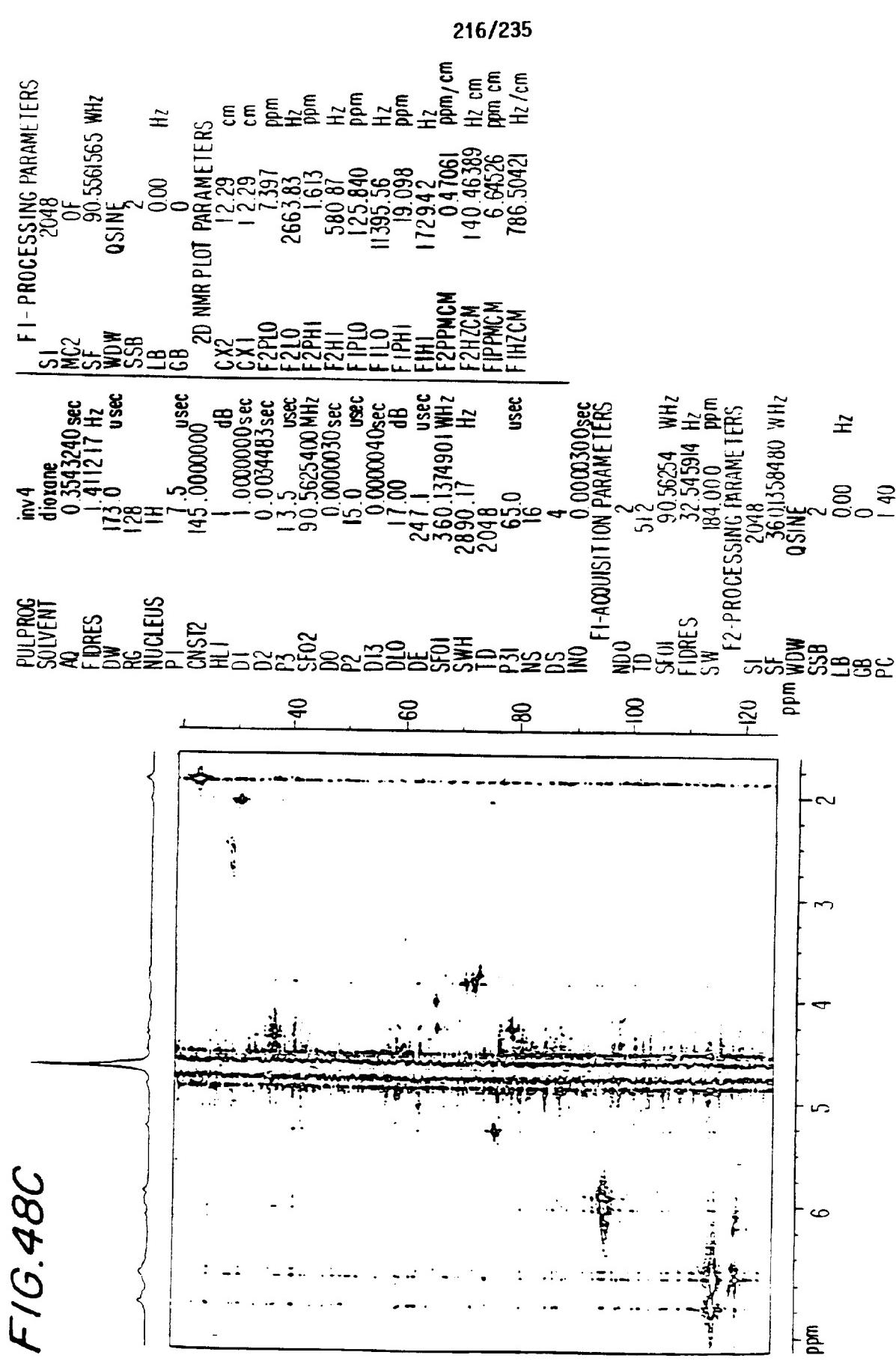
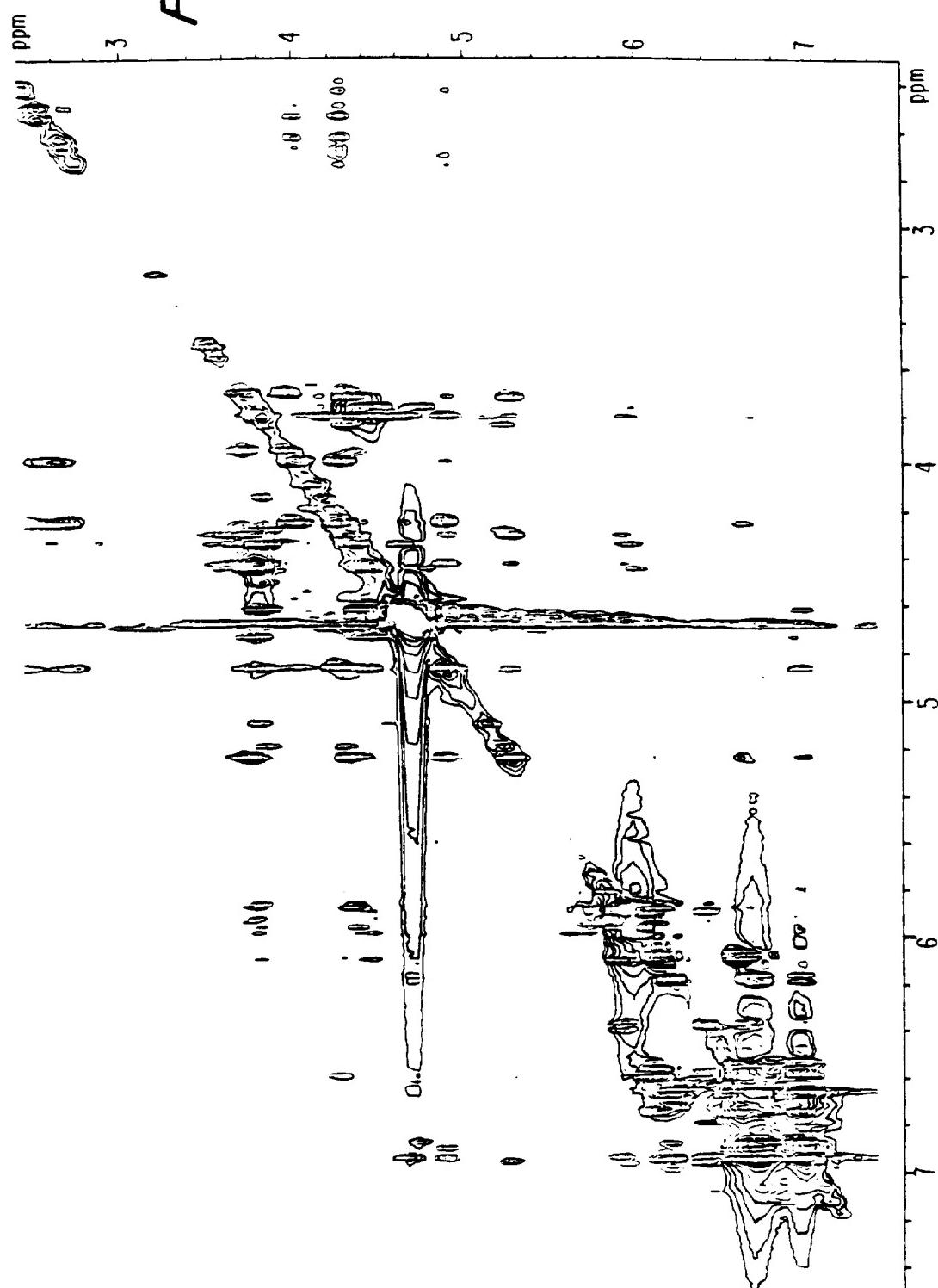


FIG. 48D

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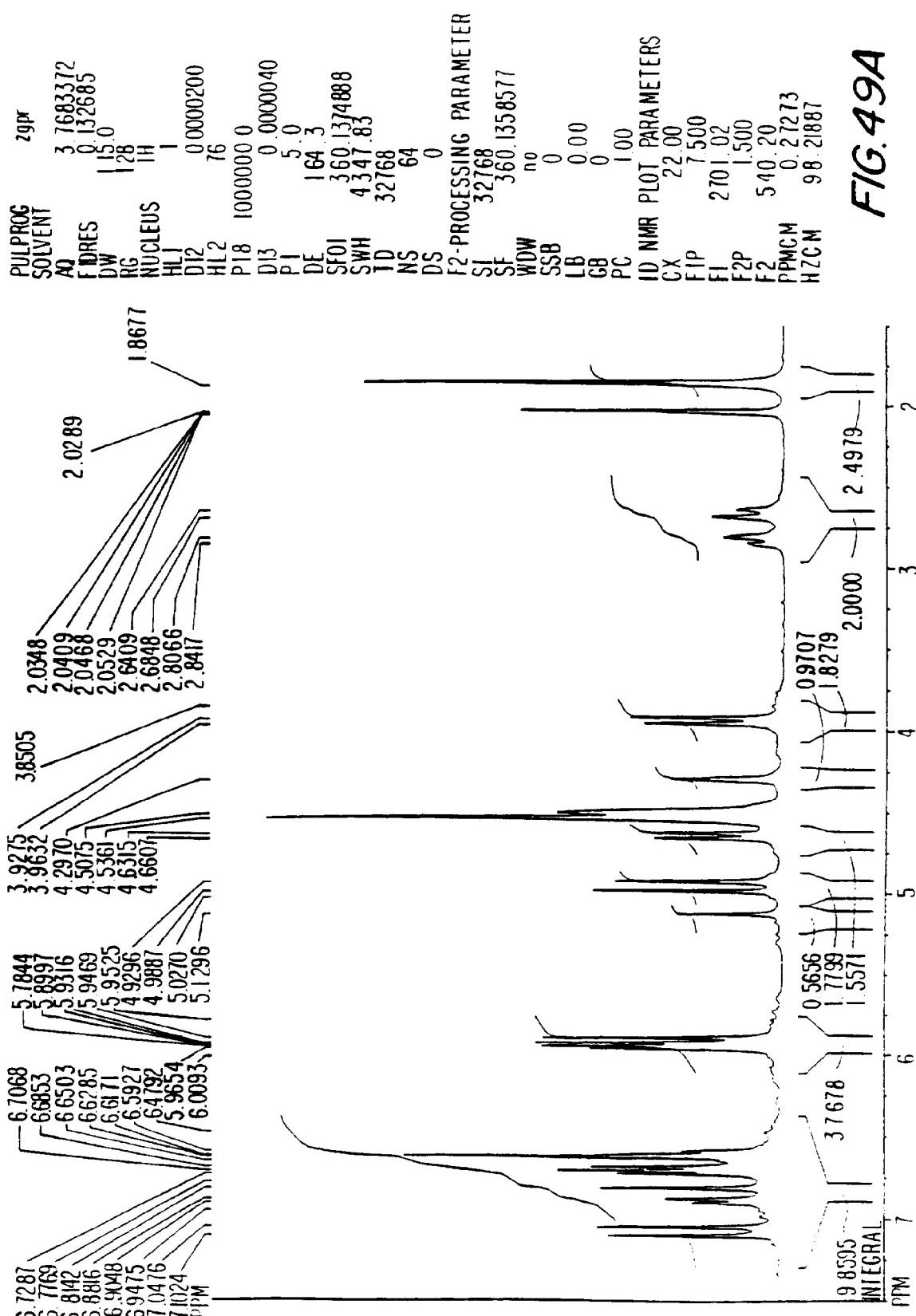
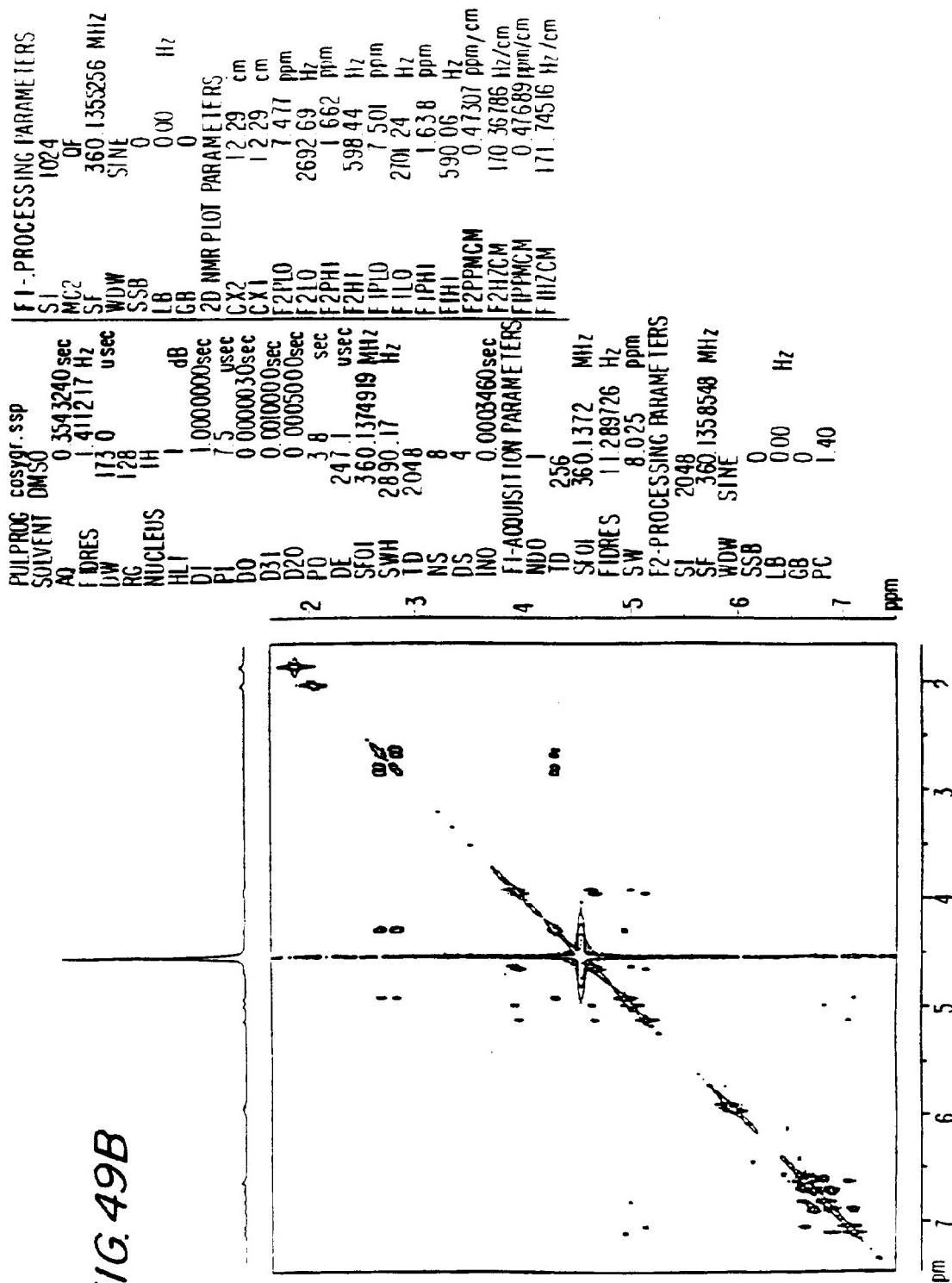


FIG. 49A

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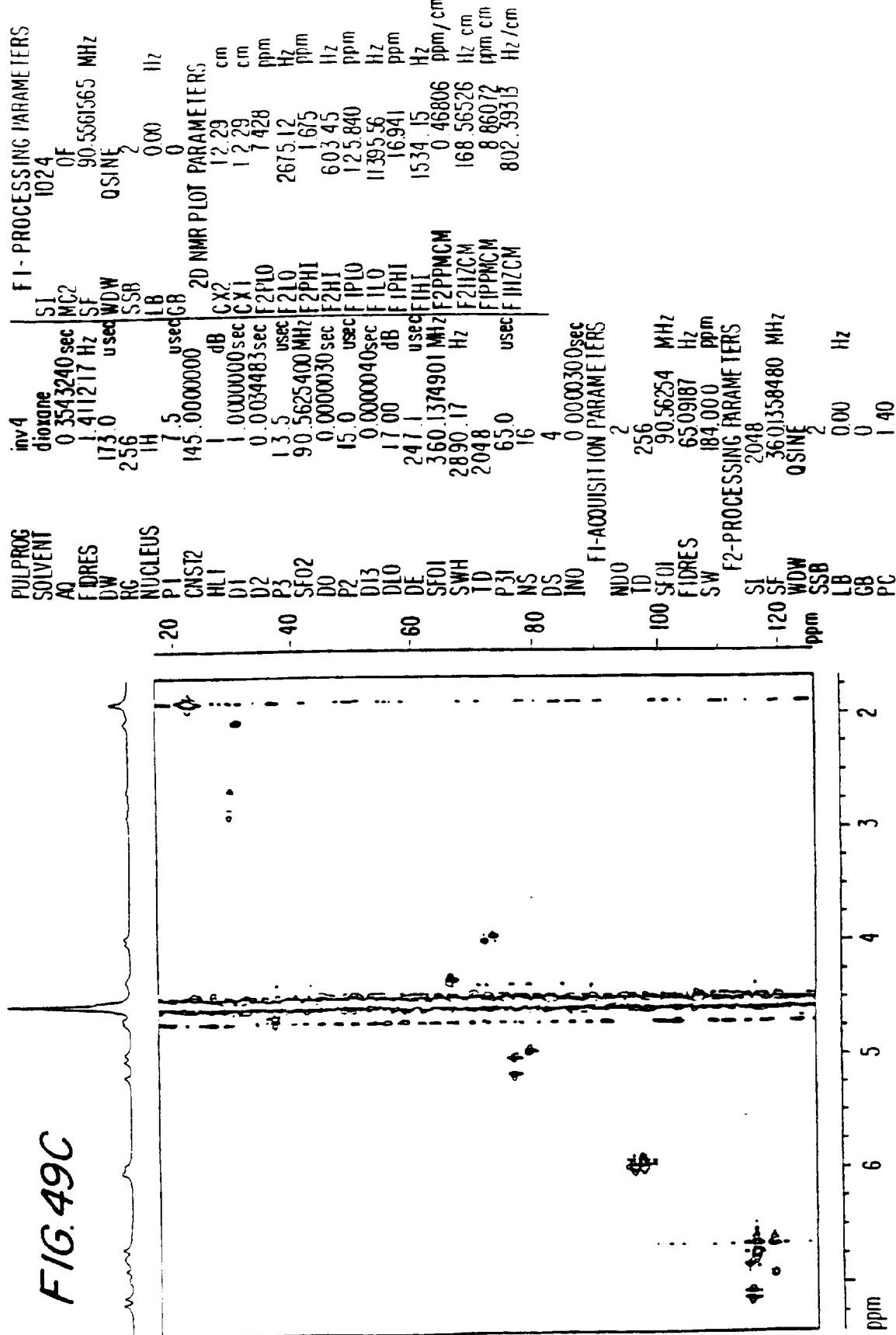
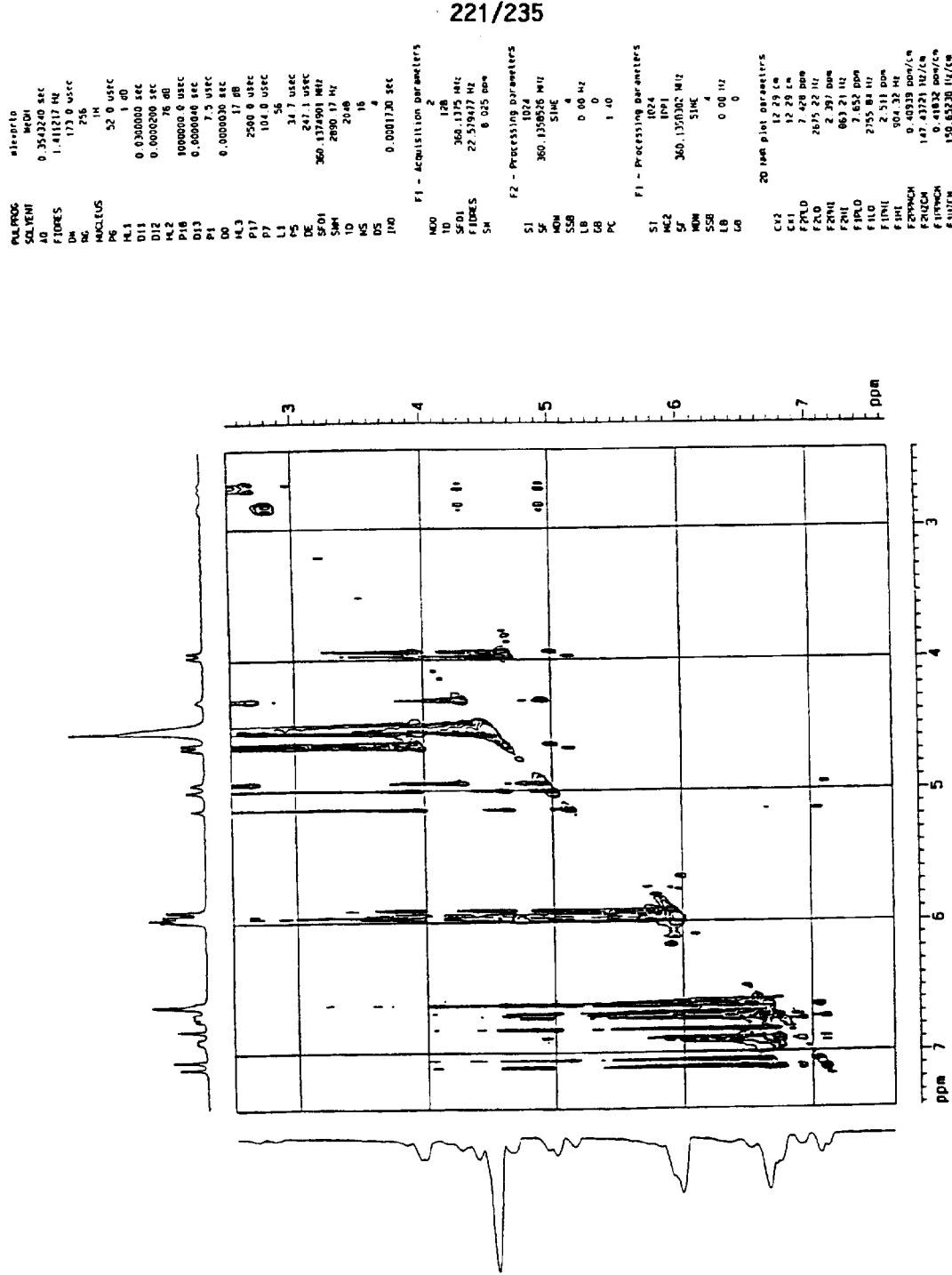


FIG. 49C

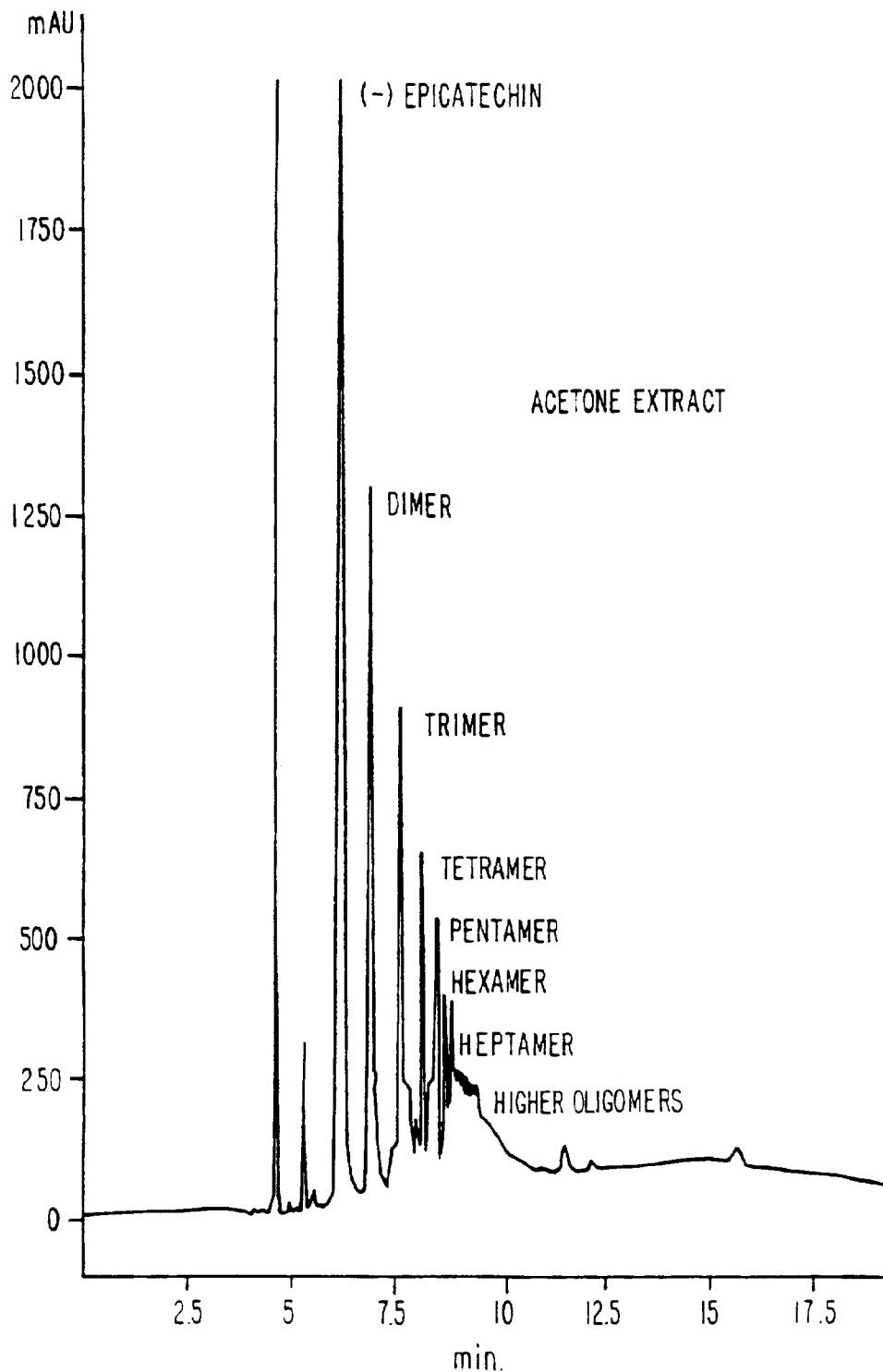
Fig. 4C1
Trimer peak 3 in 70/30 acetone-d₆/d₂O. Inverse probe ANX-360
HOHABA



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FIG. 57

DAD1 A, Sig = 200:16 Ref=450.80 of 4187\4187_1.D



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FIG. 58A

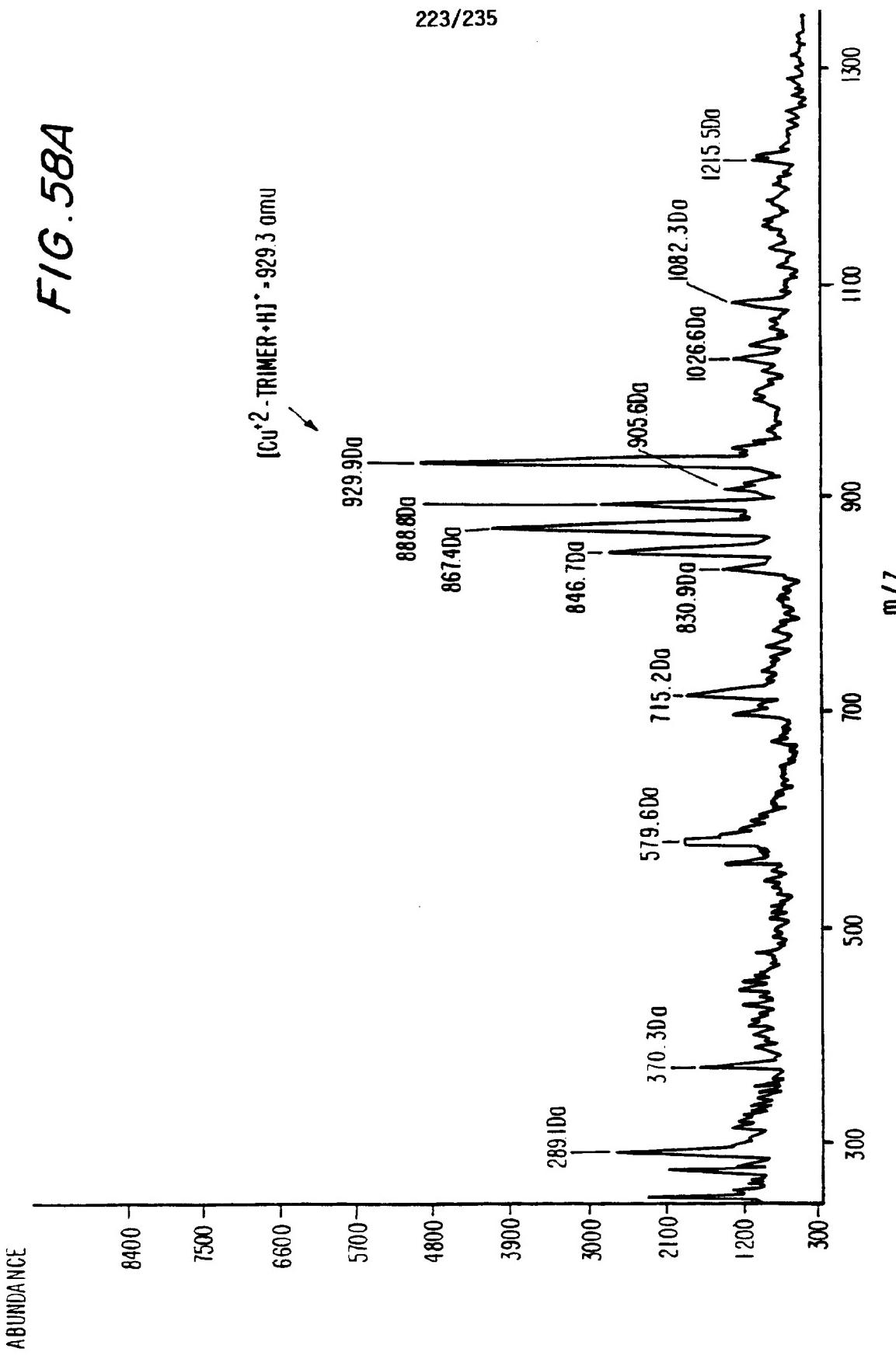
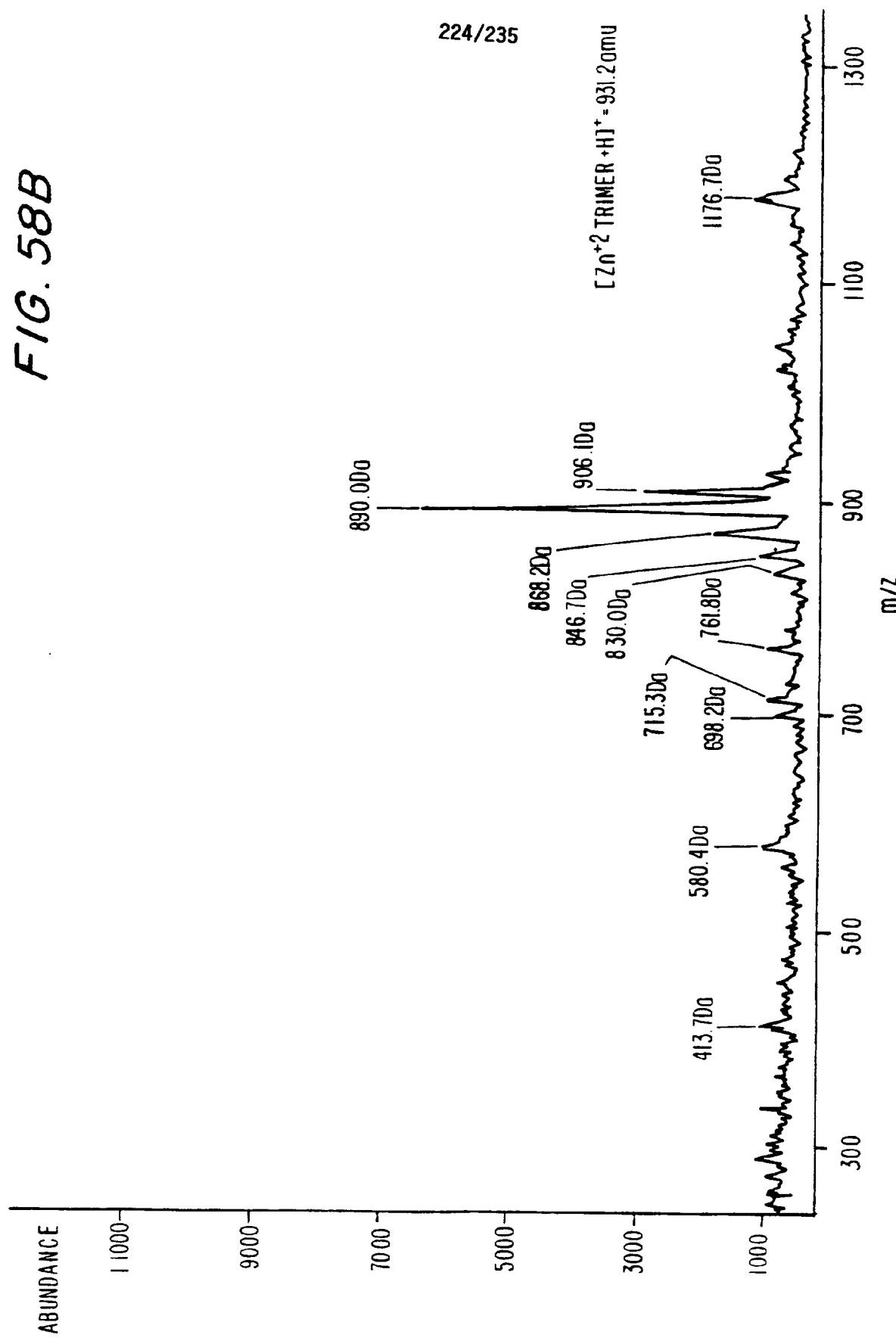


FIG. 58B

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FIG. 58C

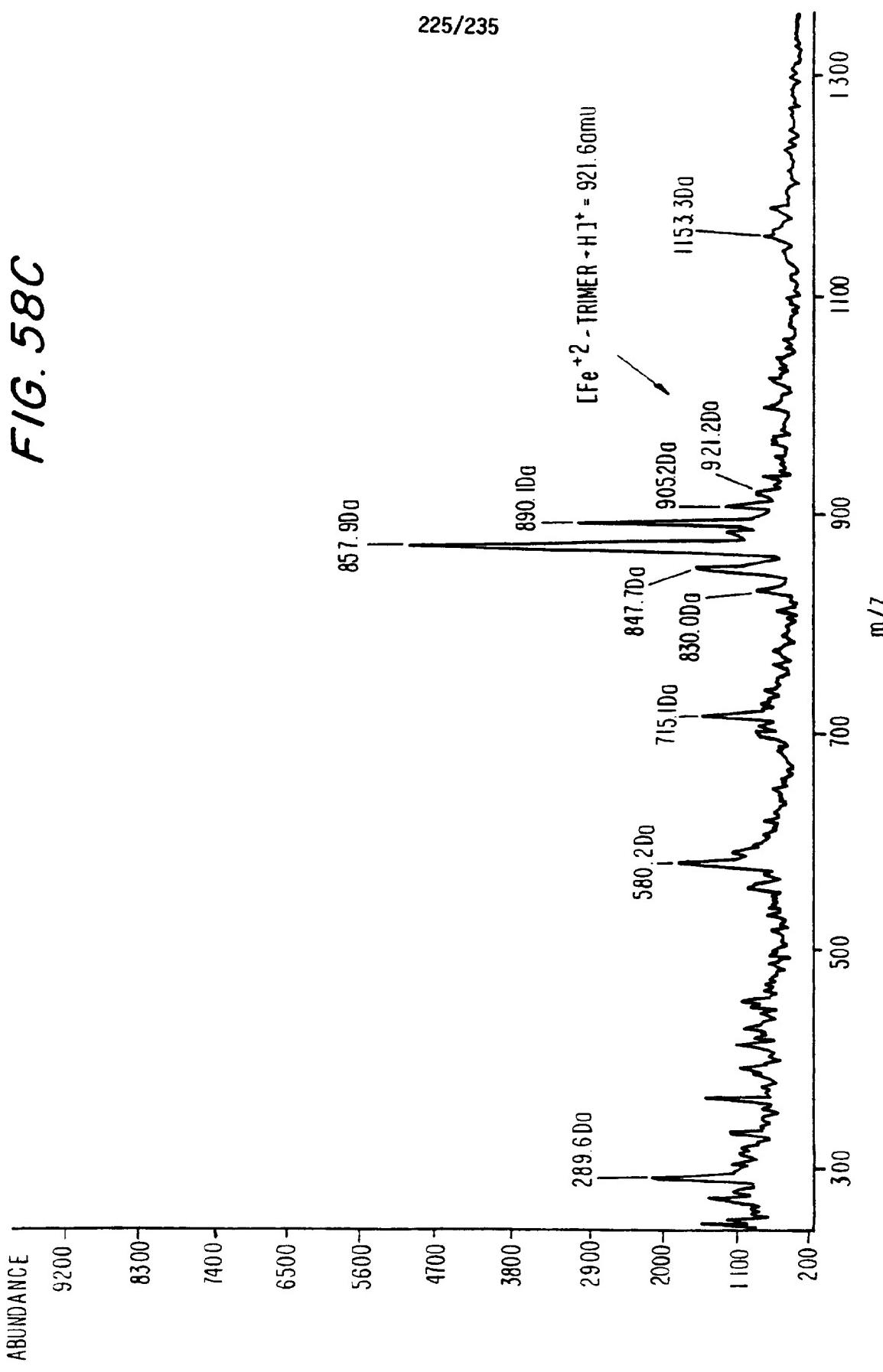
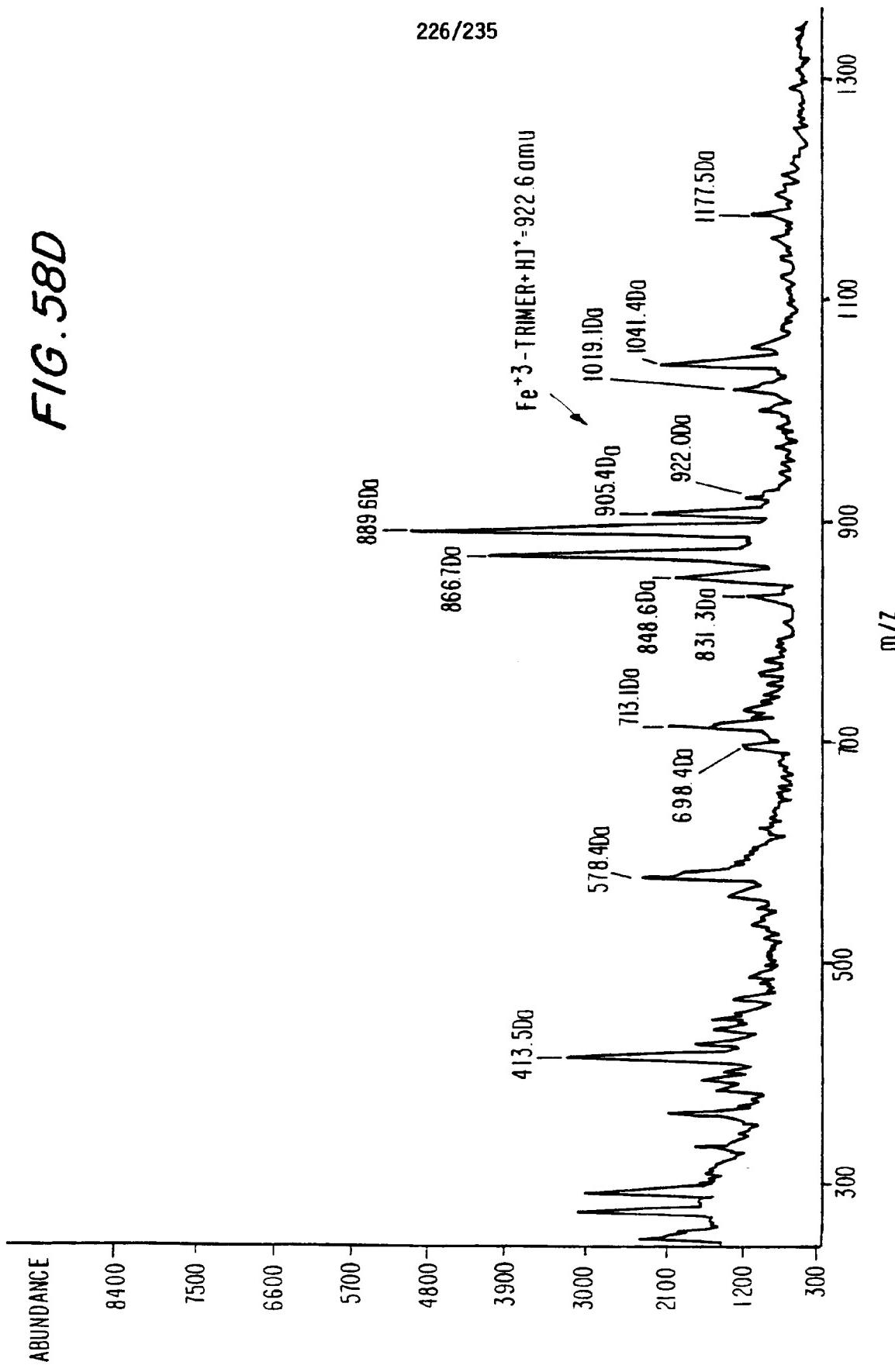


FIG. 58D

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FIG. 58E

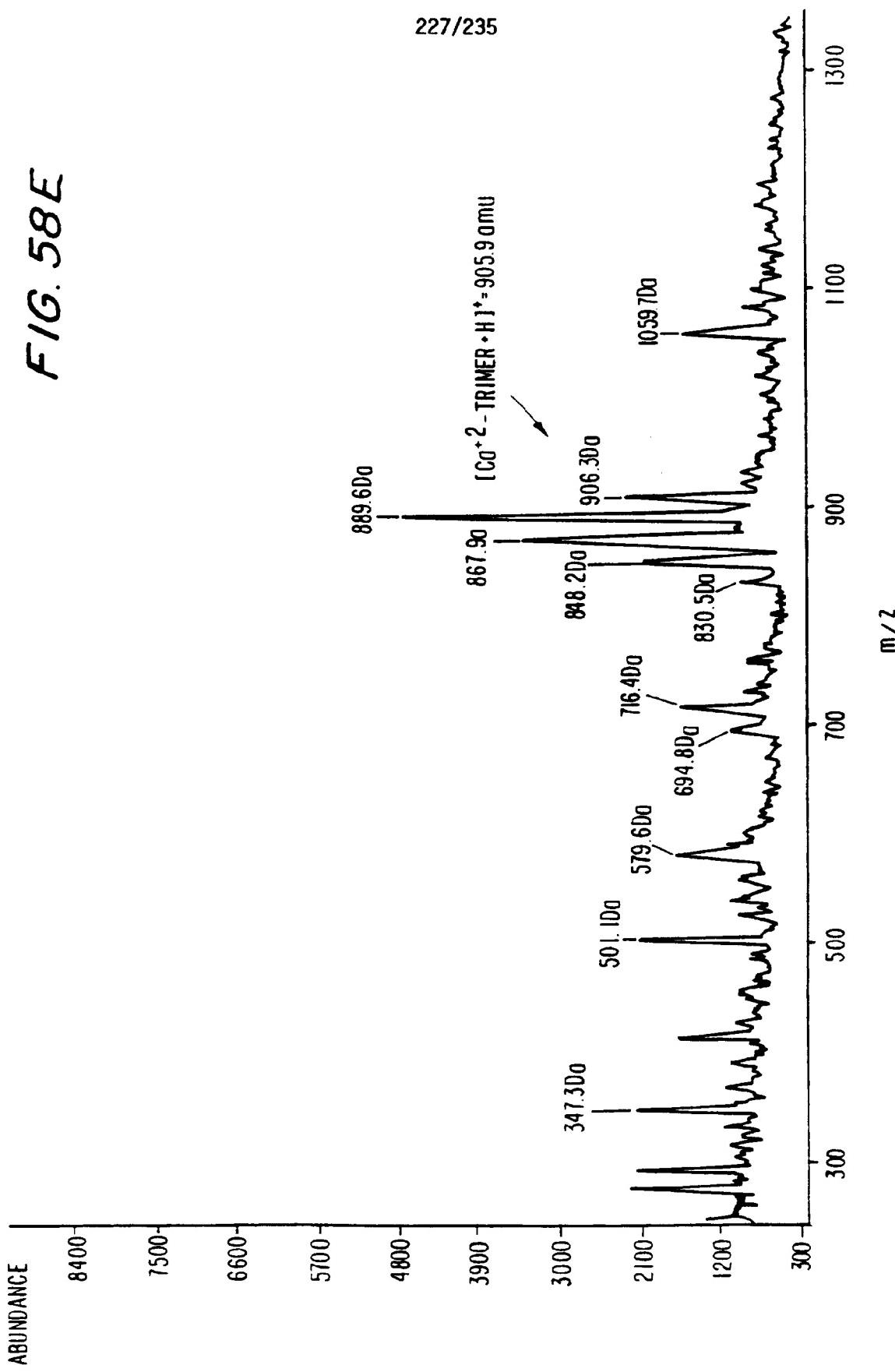
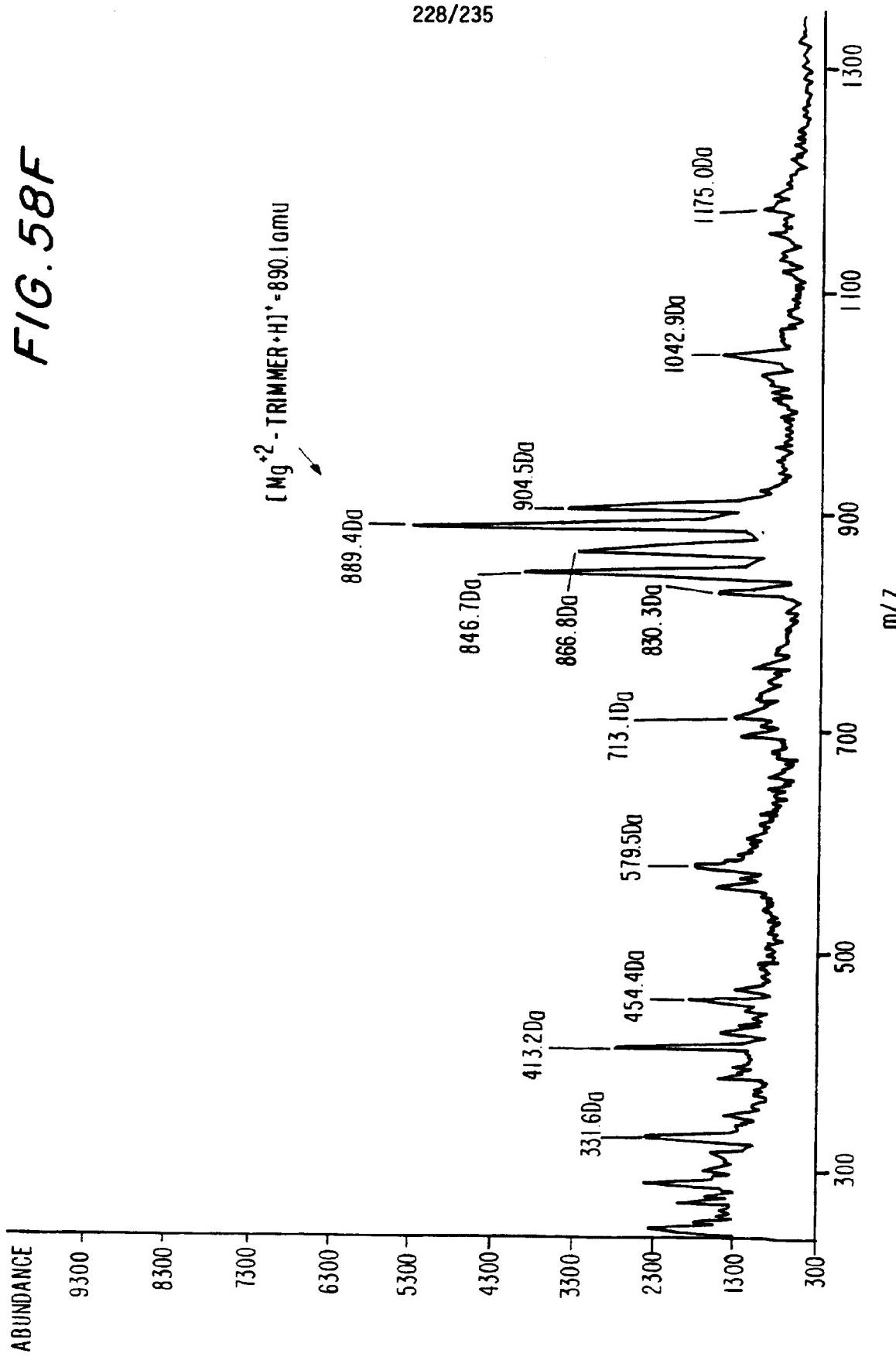
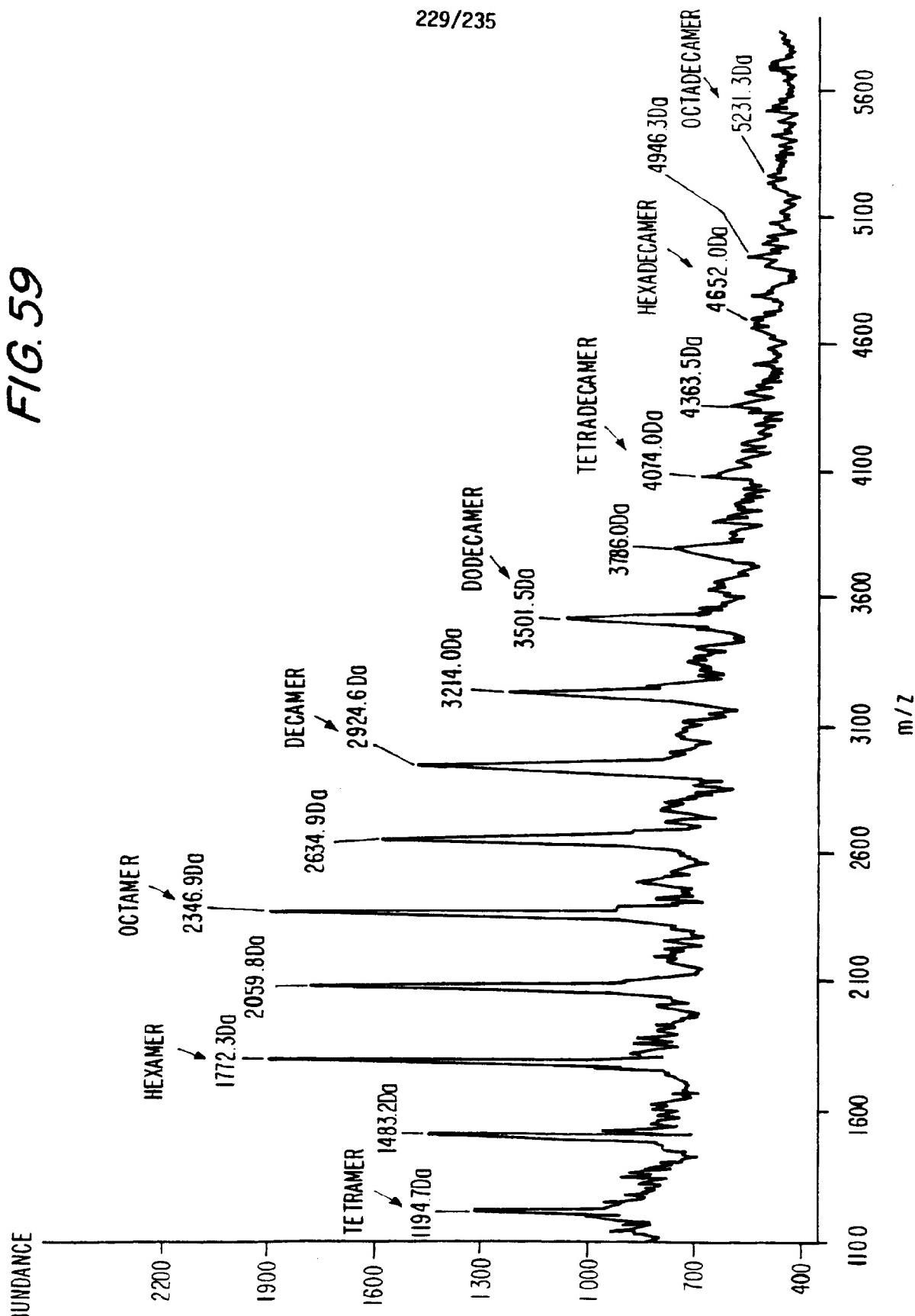


FIG. 58F



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FIG. 59



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FIG. 60

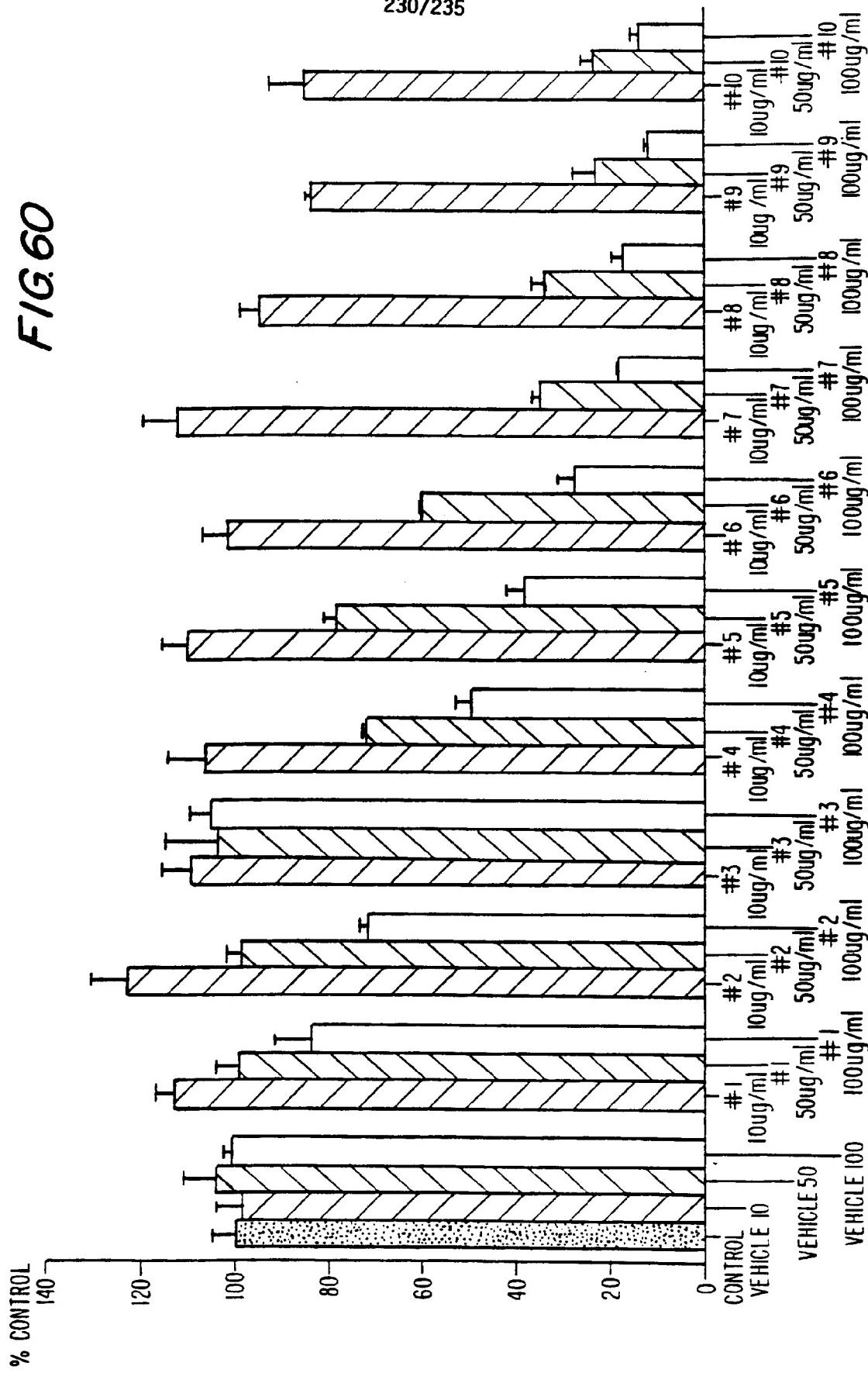
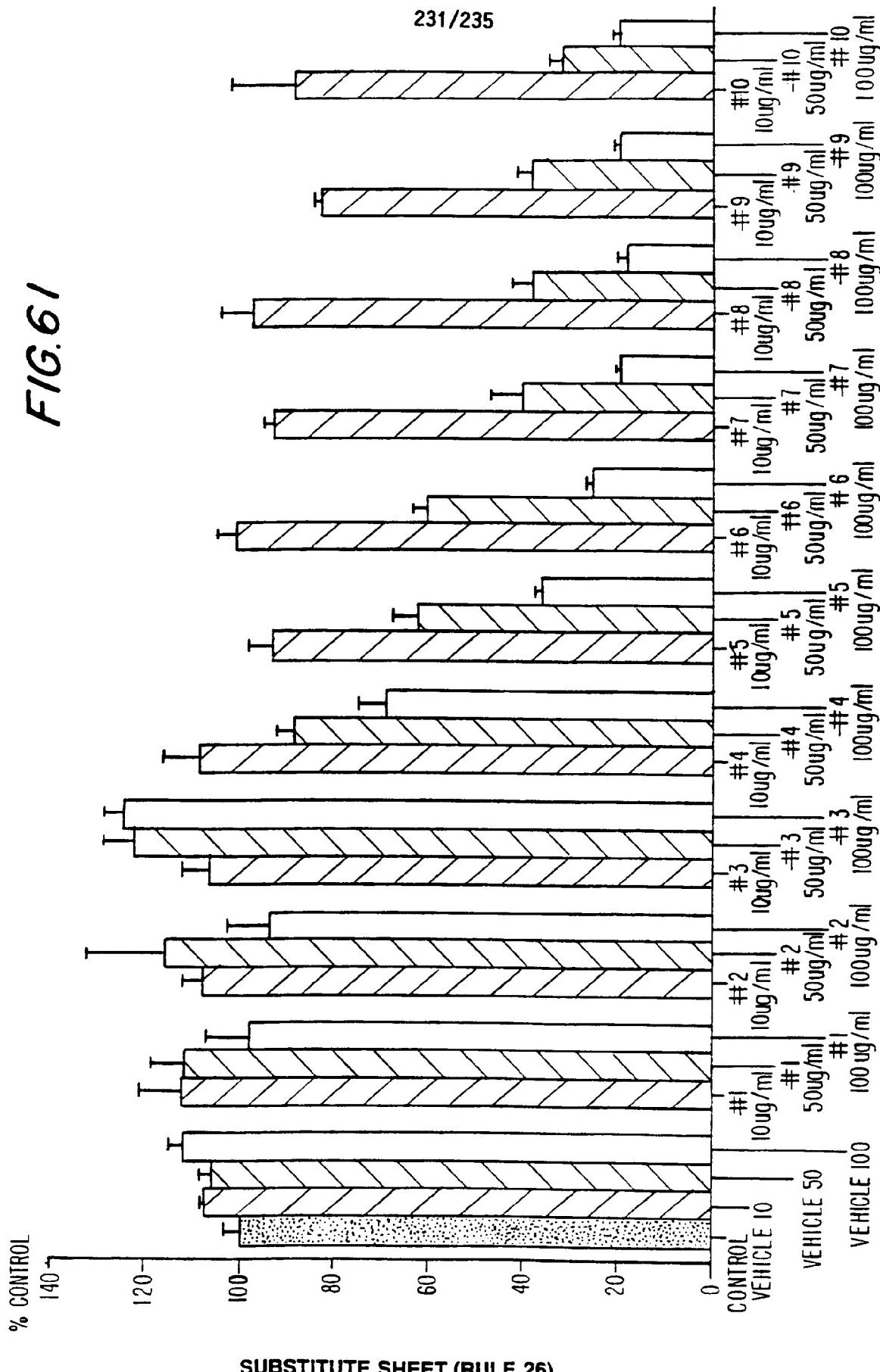


FIG. 6/



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FIG. 62

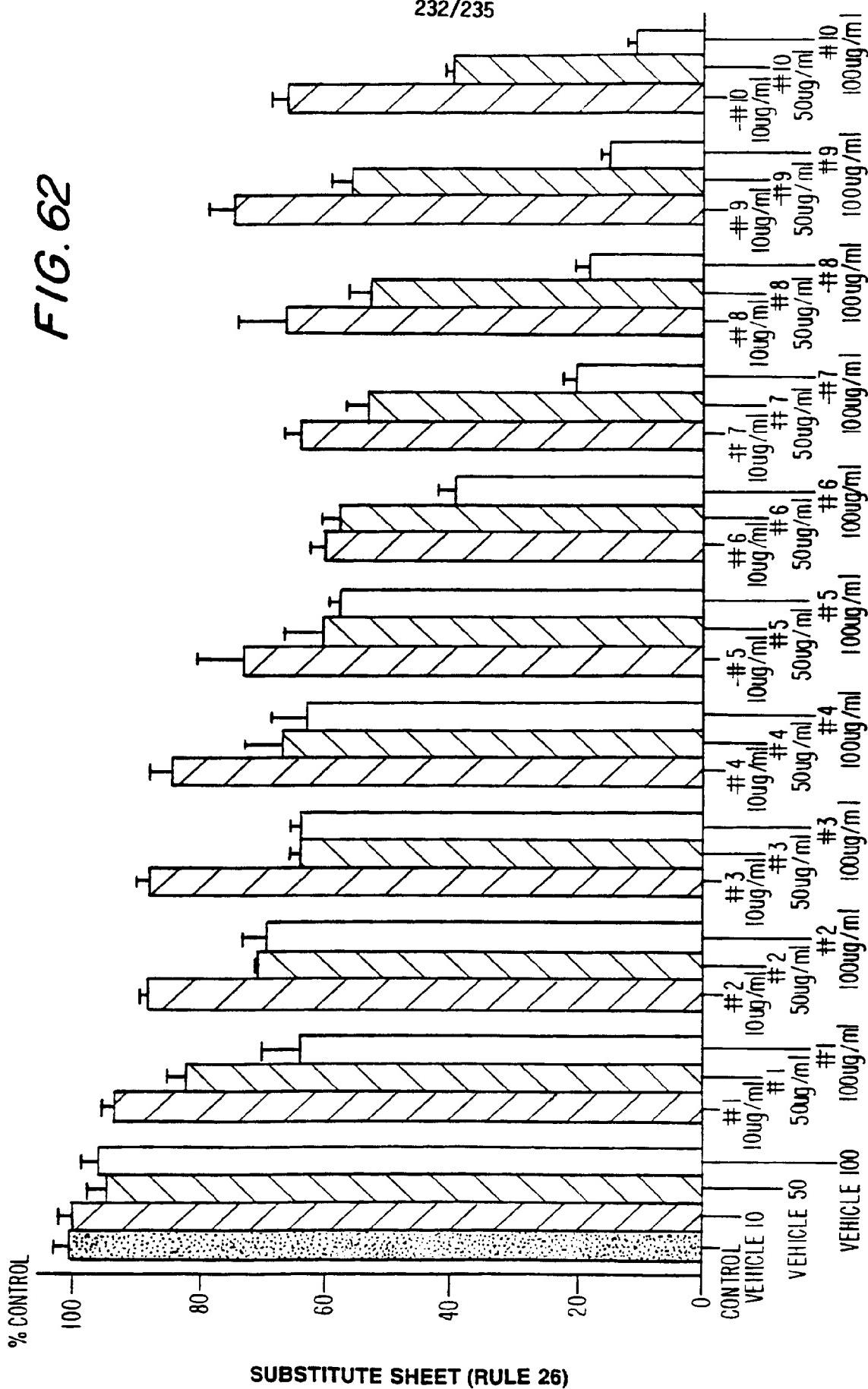
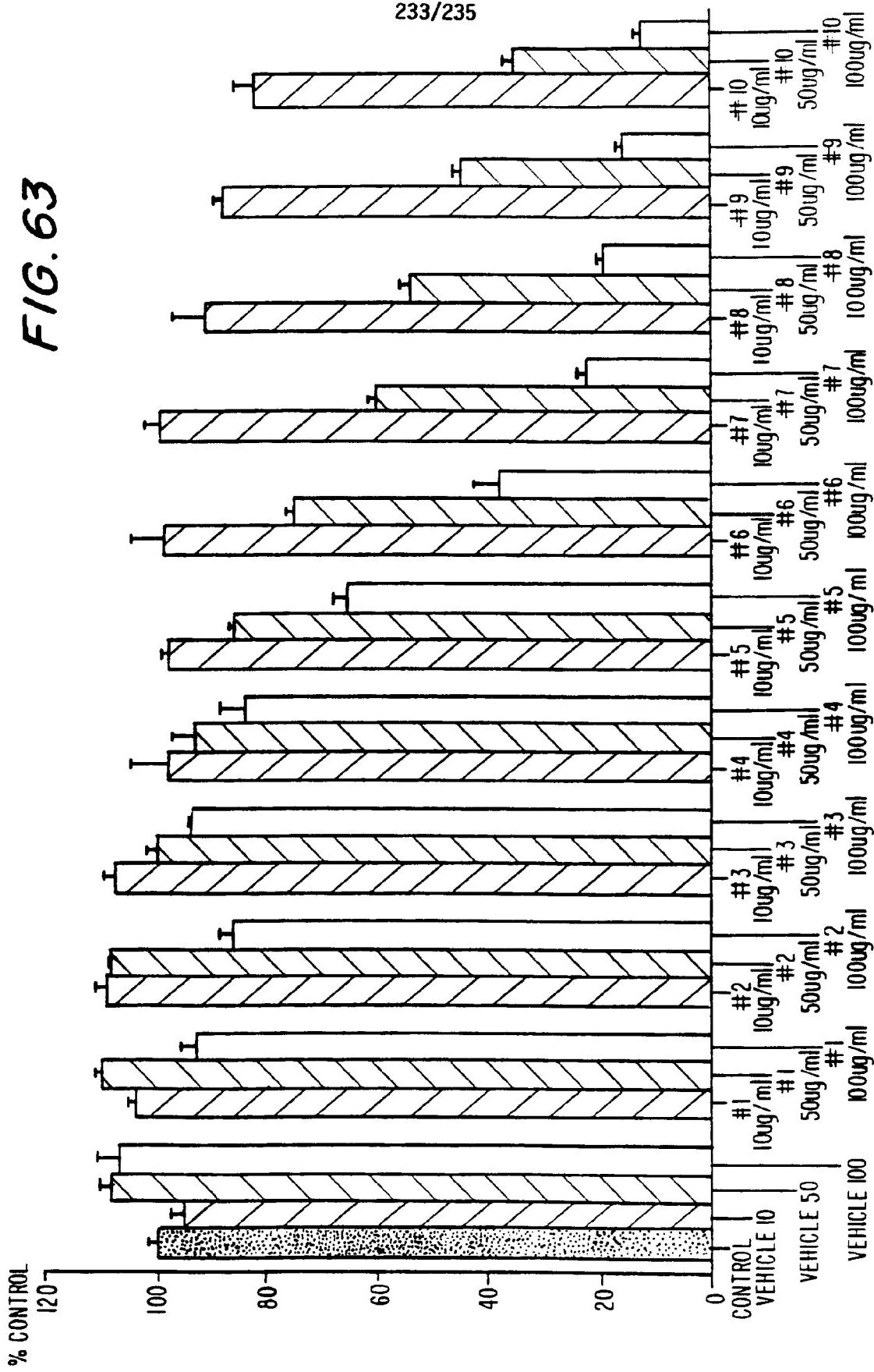
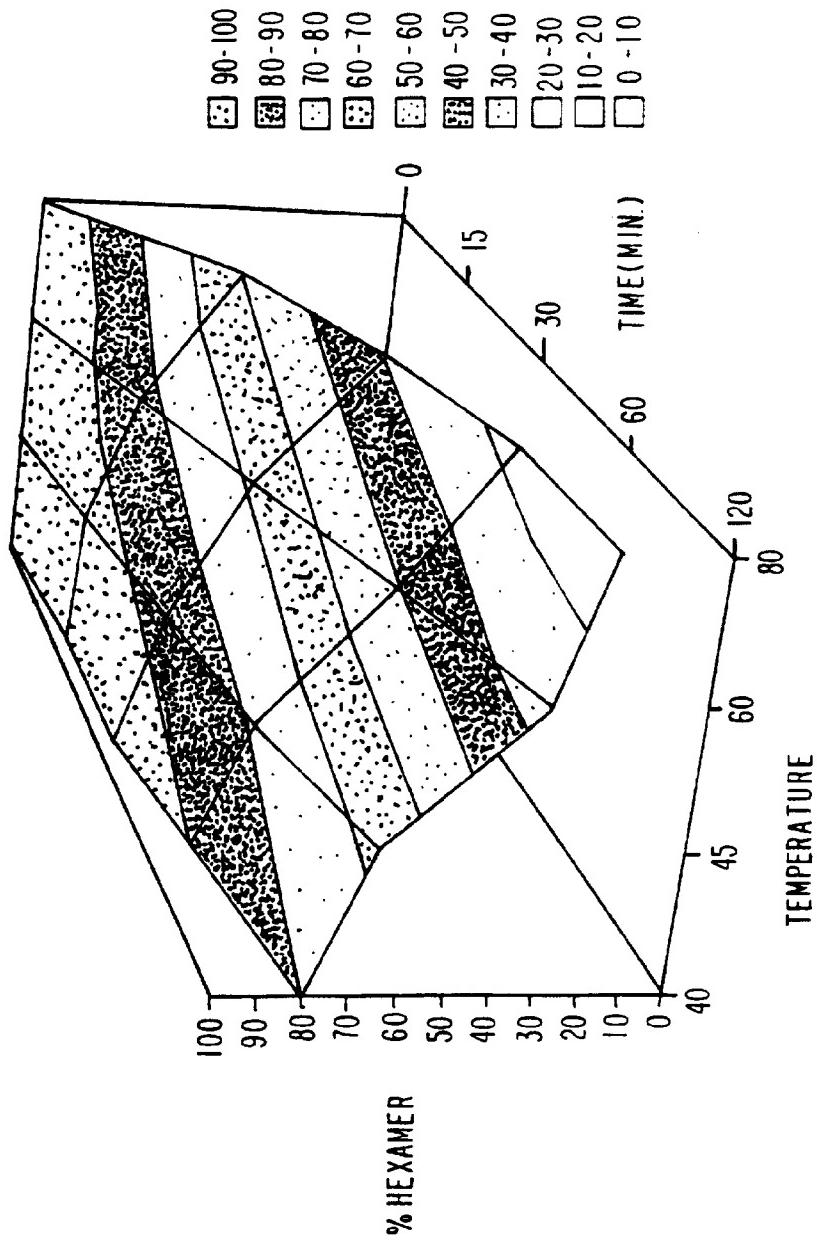


FIG. 63



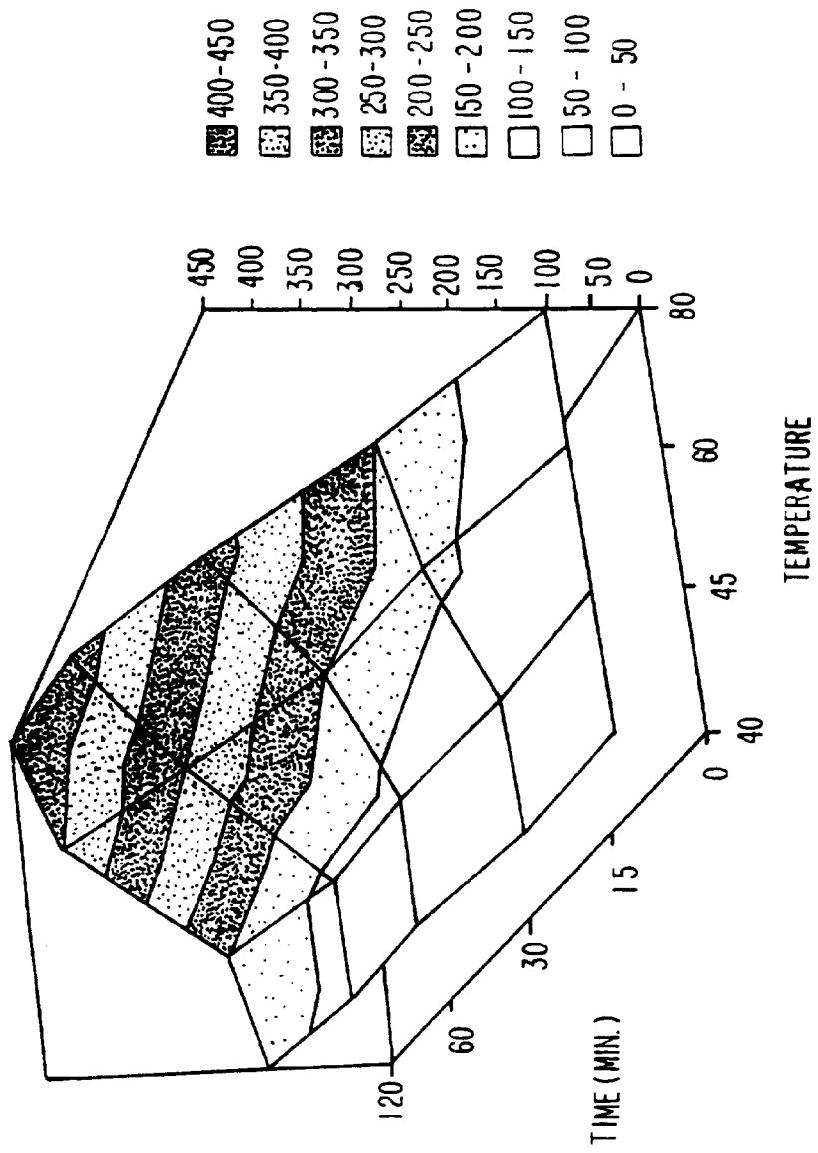
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FIG. 64



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FIG. 65



ANY REFERENCE TO FIGURES 50A-B,
51, 52, 53, 54, 55 and 56 SHALL
BE CONSIDERED NON-EXISTENT
(see Article 14(2))